The Use of Rice (*Oryza sativa* L.) in Ecotoxicological Monitoring and Toxicity Identification Evaluation (TIE)

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Under the Faculty of Environmental Studies

by

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The Use of Rice (*Oryza sativa* L.) in Ecotoxicological Monitoring and Toxicity Identification Evaluation (TIE)

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Certificate

This is to certify that this thesis entitled, **"The Use of Rice (*Oryza sativa* L.) in Ecotoxicological Monitoring and Toxicity Identification Evaluation (TIE)"** is a bonafide record of research carried out by Mr. Syamkumar R (Reg, No. 3231) under the guidance of **Dr. Rajathy Sivalingam, Professor, School of Environmental Studies, Cochin University of Science and Technology, Kochi- 682022** and co-guidance of **Dr. A. Mohandas, Professor Emeritus, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Lakeside Campus, Fine Arts Avenue, Kochi – 6082016**, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Environmental Toxicology under the Faculty of Environmental Studies, Cochin University of Science and Technology and that no part thereof has been included for the award of any other degree, diploma, associateship, fellowship, or any other similar title. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in the thesis.

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Declaration

I hereby declare that the thesis entitled, **"The Use of Rice (*Oryza sativa* L.) in Ecotoxicological Monitoring and Toxicity Identification Evaluation (TIE)"** is an authentic record of the research work carried out by me under the guidance of Dr. Rajathy Sivalingam, Professor, School of Environmental Studies, Cochin University of Science and Technology, Kochi- 682022 and co-guidance of Dr. A. Mohandas, Professor Emeritus, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Lakeside Campus, Fine Arts Avenue, Kochi – 6082016, in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy under the Faculty of Environmental Studies, Cochin University of Science and Technology and no part of this thesis has been submitted for the award of any degree, diploma, associateship, or any other title or recognition from any University/Institution.

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Syamkumar R

Dedicated to all those who are fascinated by nature.

Contents

List of Figures

List of Tables

Glossary

AI	Additivity Index
AL	Artificial Light
AVS	Acid Volatile Sulfide
BOU	Bounds of Uncertainty
CER	Cation Exchange Resin
CI	Confidence Interval
D	Darkness
EC	Electrical Conductivity
EC_x	Estimated (Effect) Concentration that causes an x% reduction in endpoint (e.g. seed germination)
EDTA	Ethylenediaminetetraacetic acid (EDTA)
FP	Filter Paper
IC_x	Inhibition concentration for (specified) percent effect.
LCI	Lower bound of Confidence Interval
LC_x	Estimated (Lethal) Concentration that causes a 50% mortality.
LOEC	Lowest Observed Effect Concentration. The lowest test concentration that is significantly different from the control.
MTI	Mixture Toxicity Index
NL	Natural Light
NOEC	No Observed Effect Concentration. The highest test concentration that is not significantly different from the control
OECD	Organisation for Economic Co-operation and Development
ORP	Oxidation Reduction Potential
PCC	Powdered Coconut Charcoal
QAC	Quaternary ammonium compounds
SDS	Sodium Dodecyl Sulfate
SLS	Sodium Lauryl Sulfate
SQG	Sediment Quality Guideline
STS	Sodium Thiosulfate
TAN	Total Ammonia Nirogen
TIE	Toxicity Identification Evaluation
TOC	Total Organic Carbon
TS	Total Solids
TU	Toxic Unit
TW	Tap Water
USEPA	United States Environmental Protection Agency

Chapter 1

General Introduction

Ecotoxicology deals with the effect of toxicants on organisms, especially at the population, ecosystem, community, and biosphere levels. One of the techniques used in ecotoxicology includes bioassay, in which a standard test species is exposed to the sample to be evaluated. Ecotoxicological

studies utilize two types of biological responses:(1) the ability of organisms to attain an endpoint such as death, growth inhibition/stimulation etc., and (2) bioaccumulation of a toxicant in the tissue (Wright and Welbourn, 2002). The terms 'indicator' and 'sentinel' are used to denote the types of organisms that show responses related to the former and the latter respectively (Beeby, 2001; Wright and Welbourn, 2002). Beeby (2001) however, demarcates 'indicators' from a third category called 'monitors' which respond to the pollutants by their impaired function/performance (in contrast to indicators which respond by presence or absence). Plants and animals have a unique ability to respond specifically to toxicants when present even below the detection limit (USEPA, 1991).

1.1 Animal Tests versus Plant Tests

General IntroductionAnimal Tests versus Plant Tests Despite being used as tools for in-situ biomonitoring and phytoremediation, plants have rarely been utilised for toxicity testing (Lewis, 1995). Most aquatic toxicity tests conducted recently utilized animals due to the wrong belief that plants are less sensitive to toxicants (Hayes, 2007). This misconception has even led some authors to suggest animals as surrogates for plants (Kenaga and Moolenaar, 1979). Recent studies with plants, however, refute this misconception (Blinova, 2004; Fairchild et al., 1998; Lytle and Lytle, 2001). Studies have also shown that the toxic response is unique to each taxon and that it is misleading to use animals as the surrogate for plants (Wang, 1990). Moreover, animals are found to be less sensitive than plants such as algae to certain toxicants (Klaine et al., 2002; Weyers

and Vollmer, 2000; Weyers, Sokull-Klüttgen, et al., 2000). Further, it should not be overlooked that plants are the primary producers and that any effect of toxicant on plant community will ultimately be manifested in animal community. Additionally, phytotoxicity data were found to be more valuable than animal toxicity data based on histopathology, physiology, and behaviour (Lewis, 1995).

A detailed review of toxicity tests with plants, especially vascular plants, has been given by (Wang, 1991). In his review, the author stresses the importance of complementing animal tests with plant tests. He further warns about the misinterpretation of results from animal tests by giving an example of a compound (Silvex) which was found to be non-toxic to *Daphnia*, but highly toxic to plants.

1.2 Single Species Tests versus Multispecies Tests

For the past few decades, the ecotoxicity studies were overly dependent on single species toxicity tests, a situation still prevails in many countries. Although studies have shown that results from single species toxicity tests can easily be related to effects at community level (Coutris et al., 2011; Guckert et al., 1993; Maltby et al., 2000; Schroer et al., 2004), they are not fool proofs to support the reliability of single species tests in predicting impacts at community level. Such results remain unchallenged due to the scarcity of dependable tests for higher levels of organisation to check the reliability (Cairns, 1984). This situation warrants the development of standard toxicity methods for higher levels of organisation especially

those including plants.

1.3 Lower Plants versus Higher Plants: Importance of Aquatic Macrophytes

The only plant group that has widely been used in toxicity tests are algae, of which very few species dominate in the literature. Many of the reports on phytotoxicity to algae are centred around *Pseudokirchneriella subcapitata*, also known as *Selenastrum capricornutum* (Lewis, 1995; Wang, 1991). The main use of algal toxicity tests has been in connection with the compliance of commercial chemicals (Klaine et al., 2002) in accordance with TSCA (Toxic Substances Control Act) and FIFRA (Fungicide and Rodenticide Act). No phytotoxicity data exists for many municipal and industrial effluents, hazardous wastes, and polluted sediments (Klaine et al., 2002). Algae and macrophytes respond differently to fluctuations in nutrient load in water bodies. For example, conditions that lead to eutrophication may be stimulatory to algae, but it can be inhibitory to macrophytes due to the toxicity of allelochemicals produced by algae (Wang, 1991). The decline in macrophytes may, in turn, affect the associated fauna in the water body. Algal tests are not suitable for bioassays with effluents that are turbid and that show temporal changes in toxicity (Wang, 1991). Wang (1990), in his study, has found that the algae were 20 % less sensitive than higher plants in detecting chemicals that elicit responses unique to vascular plants.

Considering the importance of higher plants in toxicity tests, some agencies like EC (2007), USEPA (1996), ISO (2005), and OECD (2006)

have incorporated *Lemna* tests in standard toxicity tests. In addition, *Lemna* test has effectively been used for the detection of human pathogens like *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Zhang et al., 2010) indicating its potential to be used as an early warning assay. *Lemna* is also used to in toxicity tests of effluent (Radic et al., 2010), surface water (Radić et al., 2011), sediment (Burton Jr. et al., 1996), and landfill leachate (Kalčíková et al., 2011). *Lemna* tests use classical endpoints like wet or dry weights, counts or area of fronds (7-day test). Although root elongation was found to be the most sensitive endpoint, it is difficult to measure the roots of *Lemna* due mainly to their delicacy (Davis, 1981). Recently, a method suggested by Park, Kim, et al. (2013) has overcome this problem; this method measures the re-growth (post-exposure) of roots removed before the toxicant exposure. It also has an additional advantage that it requires shorter duration (48-h) and smaller volume (3 ml) of test solution than those required for standard methods. *Lemna gibba* and *L. minor* are the commonly used test species of Lemnaceae family. Another important macrophyte genus used in toxicity tests is *Spirodela*. In addition to the traditional endpoints used, a recently developed toxicity test using turion (dormant buds for surviving harsh conditions) formation is also gaining attention in the case of *Spirodela* (Oláh et al., 2016). Both *Lemna* and *Spirodela* are known as duckweeds. Recent advancements (e.g., genome sequencing) in molecular biology have made it possible to diagnose toxicants via DNA-microarray based profiling of gene expression in duckweeds (Ziegler et al., 2016).

It is noteworthy that macrophyte toxicity data available at present

abounds with those from floating macrophytes like *Lemna*. Rooted submerged and emergent macrophytes are infrequently used in toxicity tests because of their large size, gentle growth and unavailability of standard test methods (Lewis, 1995). The fact that the entire plant body and roots of macrophytes are in direct contact with toxicants makes them a valuable tool in toxicity assessment of environmental samples, especially sediments (Lewis, 1995). It should also be mentioned that it is unrealistic to use duckweeds in sediment bioassays as they are floating plants and are exposed to toxicants only through their lower frond-surface (Sánchez et al., 2007). Lewis (1995) in his review on the use of freshwater plants in toxicity testing, has suggested a number of macrophytes that could be used in toxicity assessment (Table 1.1). More recently, ISO (2013)and OECD (2014) standardised the test methods (the sediment toxicity tests) for *Myriophyllum aquaticum* and *Myriophyllum spicatum*, respectively. *Myriophyllum* has been shown to be sensitive to some plant protection products (Deneer et al., 2013; Mohr et al., 2013; Tunić et al., 2015) and metals (Sánchez et al., 2007). It is important to note that *Myriophyllum* tests, like *Lemna* tests, also require nutrient the medium. Studies have shown that the composition of test solution may interfere with the test results (Huebert and Shay, 1992; Wang and Freemark, 1995).

Most toxicants are found to accumulate in sediments, some of them are at much lower concentrations. Herbicide concentrations that are generally found to be less toxic to most macrophytes may reflect their detrimental effect indirectly at the community level (Coutris et al., 2011). Such indirect effects are the results of varied sensitivity among species to

toxicants, which disrupts the pattern of interspecific interaction (Relyea and Hoverman, 2006).

1.4 Influence of Duration in Toxicity Tests

Exposure duration is an important factor which is rarely been given much importance in many toxicity tests (Newman and McCloskey, 1996). In most toxicity studies, contaminant sensitivity to different test species is compared at different test durations. However, the relative toxicity of chemicals exposed to different organisms for different durations is difficult to compare (Mackay et al., 2014). Time factor can be incorporated into toxicity tests either as 'acute' or 'chronic' timescales or as 'time-to-event' analysis (Newman and McCloskey, 1996). These methods, however, requires that the tests be performed at multiple time points. Unfortunately, most phytotoxicity studies available at present depends mainly on the single duration of exposure. Generally, shorter test durations are desirable for toxicity tests as some toxicants show change in bioavailability during the course of time (Klaine et al., 2002). Toxicity tests using the photosynthetic activity as endpoints use tests durations which extend only up to few hours or minute (Strom et al., 2009; Wang, 1994).

1.5 Variability in Toxicity Tests Using Plants

Taxonomic variability among plants species in sensitivity to toxicants was found to be high (Klaine et al., 2002). Unrealistic nature of standard test methods and wide taxonomic variability among plants in response to toxicants are the major factors that has been found to affect the predictive value of test results from single species to natural plant community

Table 1.1: List of macrophytes and effect parameters commonly used in toxicity tests (Lewis, 1995).

Test species	**Effects**
Chara hispada	Biomass
Ceratophyllum demersum	Abundance
Eichinochloa crusagalli	Chlorophyll content
Eichhornia crassipes	Enzyme activity
EIodea canadensis	Node counts
E. nuttalli	Frond counts
Hydrilla verticillata	Root length
Lemna minor	Organelle structure
L. perpusilla	Stem length
L. gibba	Seed germination
Myriophyllum spicatum	Photosynthetic activity
M. alterniforum	Seedling growth
M. brasiliense	
Najas yuadalupensis	
N. flexims	
Potamogeton pectinatus	
P. perfoliatus	
P. pectinatus	
P. coloratus	
P. illinoensis	
P. natans	
P. crispus	
P. foliosus	
P. nodosus	
Spirodela polyrhiza	
Vallisneria americana	

(Lewis, 1995). A comprehensive analysis of phytotoxicity data entered in PHYTOTOX database of USEPA has been made by Fletcher, Johnson, et al. (1988) and Fletcher, Muhitch, et al. (1985). His analyses indicated that phytotoxicity data for several chemicals were found to be scanty. Further, interspecies comparisons showed that monocots (oat and wheat) were most sensitive to several herbicides. The cucumber was found to be the most sensitive among dicots. Fletcher, Muhitch, et al. (1985) also found that no plant species was consistently sensitive to all classes of chemicals.

According to a recent study, vascular plants, especially the terrestrial ones, showed great variability in sensitivity to most chemicals (Elmegaard et al., 2000). Another review by Clark et al. (2004) showed that the variability in response to toxicants greatly increases as one moves from lower (species or family) to higher (class or order) taxon. He further, noted that the PHYTOTOX database was dominated by north-temperate agricultural species and that there was a paucity of sufficient information on grassland, coniferous forest, and desert biomes. It appears from these results that plants, due to their diversity and taxonomic variability in response towards toxicants, deserve much more attention as toxicity test species and therefore, phytotoxicity data from a variety of sources including chemicals and environmental samples are demanded.

1.6 Influence of Test Medium in Toxicity Tests

Most toxicity tests, especially those involving algae, use nutrient-rich culture medium the composition of which rarely matches with that of the

environmental samples (Lewis, 1995). This may confound the results as the components in the test solution is likely to interfere with the toxicants. For example, the excess concentrations of EDTA in the test medium was found to reduce the toxicity of Cd and Zn to *Lemna trisulca* (Huebert and Shay, 1992). Millington et al. (1988) observed unpredictable variations in sensitivity of three algal species (*Chlorella vulgaris*, *Scenedesmus subspicatus* and *Selenastrum capricornutum*) to four chemicals in toxicity tests with three test mediums (Bold's basal, EPA, and OECD media). Influence of nutrient medium on toxicity has also been observed in the case of uranium toxicity to *Lemna minor* (Horemans et al., 2016). According to Janssen and Heijerick (2003) pH, hardness, type of test medium, pre-culture conditions, and presence of chelating agents are the key factors that influence the metal toxicity to algae. Fjällborg et al. (2006) observed reduced toxicity of Ag to *Daphnia magna* in reconstituted water compared with *Lactuca sativa* in pure water. He attributes this reduction in toxicity to the formation of Ag complex in reconstituted water. It is worth to note that seed germination tests do not have this problem as they can be performed in pure water (distilled or deionised water) without added nutrients.

1.7 Seed Germination and Root Elongation Tests

Seed germination tests have several advantages when compared with other tests. The ability of seeds to remain dormant during unfavourable conditions enables us to store them for longer durations. Besides this,

seed germination tests are cost effective, and versatile in nature. These tests can be used to evaluate the toxicity of both liquid (e.g. effluents) and solid (e.g. sediments) samples (Wang, 1991). One unique advantage of bioassays involving seed germination and root elongation is that they can be run with and without light so that photosensitive toxicants can easily be detected in the samples (Wang, 1991).

The utility of seed germination tests in sediment toxicity tests have been evaluated by Baran and Tarnawski (2015) who compared the performance of different test kits, Phytotoxkit and Phytotestkit (*Sorghum saccharatum*, *Lepidium sativum*, and *Sinapis alba*), Ostracodtoxkit F (*Heterocypris incongruens*), and Microtox®(*Vibrio fischeri*). The result indicated that plant tests were more sensitive than animal tests with regard to solid phase and whole sediment toxicity. Seed germination tests could also capture effects such as hormesis (biostimulation) which animal tests usually fail to detect. For example, sediments from Lake Orta have been shown to be stimulatory to *Lepidium sativum* and *Lactuca sativa*, but inhibitory to animals (Rossi and Beltrami, 1998). In later studies, indices derived from seed germination have been successfully employed in preparing phytotoxicity maps of the Lake Orta (Barbero et al., 2001).

Allium test has been found to be an encouraging option with regard to toxicity assessment of environmental samples (Fiskesjö, 1985, 1988). Due to its ease of availability, suitability as a short-term test tool, cost effectiveness, and relevance in chromosomal studies, *Allium* has been widely suggested as a standard test species. A list of species generally used in seed germination and/or root elongation tests with environmental

Table 1.2: Species used in seed germination and/or root elongation tests with different environmental samples.

Species	Sample	Reference
Amaranthus hybridus	Effluent	1
Allium spp.	Effluent	2,3,4
	River/stream water	5, 6, 7, 8, 9,
	Sediment/sludge	10,11
Cucumis spp.	River/stream water	9
Lactuca sativa	Effluent	12,13
	Soil	14
	Sediment/sludge	15
Lepidium sativum	Sediment/sludge	16,17,18
Linum usitatissimum	Sediment/sludge	19
Panicum spp.	Effluent	20
	River/stream water	9
	Soil	21
Rhaphanus spp.	Soil	21
Scirpus robustu	Sediment/sludge	22
Sinapis alba	Sediment/sludge	16
Sorghum saccharatum	Sediment/sludge	16,17
	Sediment/sludge	16,17
Spartina alterniflora	Sediment/sludge	22
Trifolium pratense	Soil	21
Triticum aestivum	Soil	21
Typha latifolia	Sediment/sludge	23
Vigna radiata	Effluent	2

1 (Odjegba and Oyenekan, 2016); 2 (Haq et al., 2016); 3 (Matsumoto and Marin-Morales, 2004); 4 (Pathiratne et al., 2015); 5 (Athanásio et al., 2014); 6 (Arambašić et al., 1995); 7 (Egito et al., 2007); 8 (Kenady, 1998); 9 (Siddiqui et al., 2011); 10 (Bolsunovsky et al., 2016); 11 (Geras'kin et al., 2011); 12 (Park, Yoon, et al., 2016); 13 (Priac et al., 2017); 14 (Bagur-González et al., 2011); 15 (López-Gastey et al., 2000); 16 (Baran and Tarnawski, 2015); 17 (Czerniawska-Kusza and Kusza, 2011); 18 (Barbero et al., 2001); 19 (Mamindy-Pajany et al., 2011); 20 (Wang and Williams, 1989); 21 (Banks and Schultz, 2005); 22 (Lewis et al., 2001); 23 (Muller et al., 2001)

samples is given in the Table 1.2.

1.8 Influence of Test Substrates in Seed Germination and Root Elongation Tests

Plant seeds, especially the terrestrial ones are not adapted to germination in aqueous media and hence, they need growth substrate for their normal germination. Historically, filter paper has been used for seed germination in which seeds were held on a filter paper placed either as a single layer against a flat substratum (Konzak et al., 1976) or as a sandwich in a rack (Edwards and Ross-Todd, 1980; Myhill and Konzak, 1967); discs of filter paper saturated with test solution has also been used as the growth substrate (Swanson, 1946). Ratsch and Johndro (1986) compared the toxicity of six compounds to lettuce seed germinated on filter paper with those germinated in glass bulbs aerated with compressed air (Fig 1.1). They observed that out of the six compounds tested, five (monosodium methanearsonate, $AgNO_3$, $CdCl_2$, monuron, and 2,4-D) were required in smaller concentrations for glass bulb than those required for filter paper method to cause toxicity to root. Reduced toxicity of some compounds on filter paper is mainly due to the adsorption of toxicants on to it.

As filter paper can absorb some toxicants, it may underestimate the toxicity of certain compounds. Also, filter paper may stimulate the root growth or cause the root to adhere to it in the presence of some compounds (Wang, 1993). Wang (1993) compared the sensitivity of rice seeds to selected toxicants using filter paper, Growth Pouch-TM (a commercially available product used for testing plant seed responses) and seed tray

methods (a plastic receptacle positioned inside a Petri plate) and found that seed tray gives better results than others (Fig 1.2). The seed tray method offers the advantage that the measurement can be made easily as the roots grow vertically. Contrastingly, in Petri plate method the plant root spreads horizontally, which makes measurement difficult to perform. Although seed tray comes in handy in toxicity tests as it does not interfere with test substances, it sometimes causes the roots of seedlings to break off or to remain on the upper surface of the tray (Wang, 1993). Filter

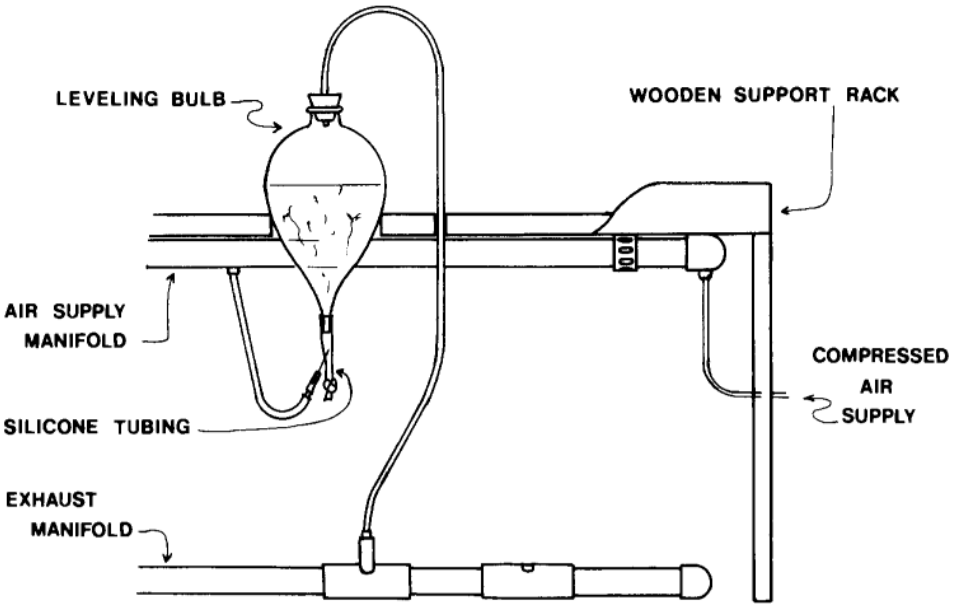

Figure 1.1: Seed germination device described by Ratsch and Johndro (1986). Seedlings remain suspended in the nutrient solution inside the levelling bulb.

paper method has also been compared with agar plate method which was found to be promising with regard to sensitivity to toxicants (Di Salvatore

et al., 2008). Besides filter paper, nylon mesh kept floating in a beaker filled with test solution was also used as a growth substrate (Wong and Bradshaw, 1982).

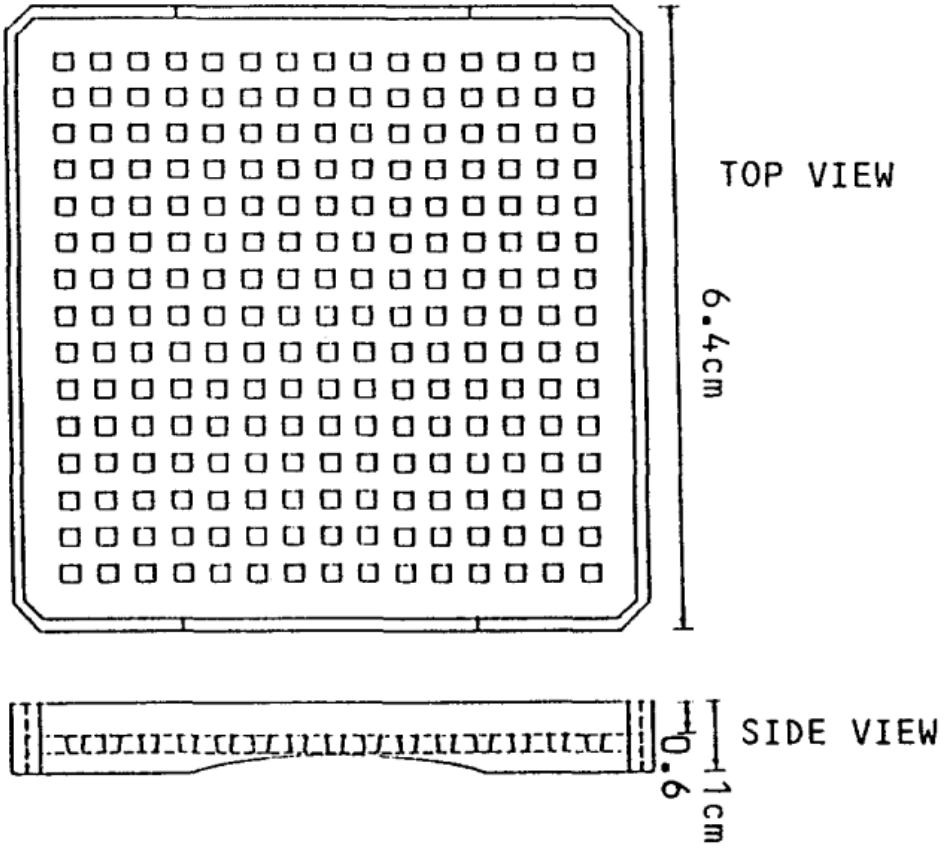

Figure 1.2: Seed tray as described by Wang (1993). Roots grow towards the test solution through the pores on the seed tray.

Recently, Andersohn et al. (2002) developed a time-saving method for phytotoxicity tests in which he devised non-sterile cotton gauze placed

on styropor pellets floating in a solution to germinate seeds (Fig 1.3). However, this method is not easy and time-saving as it claims because it requires additional support materials (cotton gauze and styropor pellets) and higher volume of test solution compared to Petri plate method.

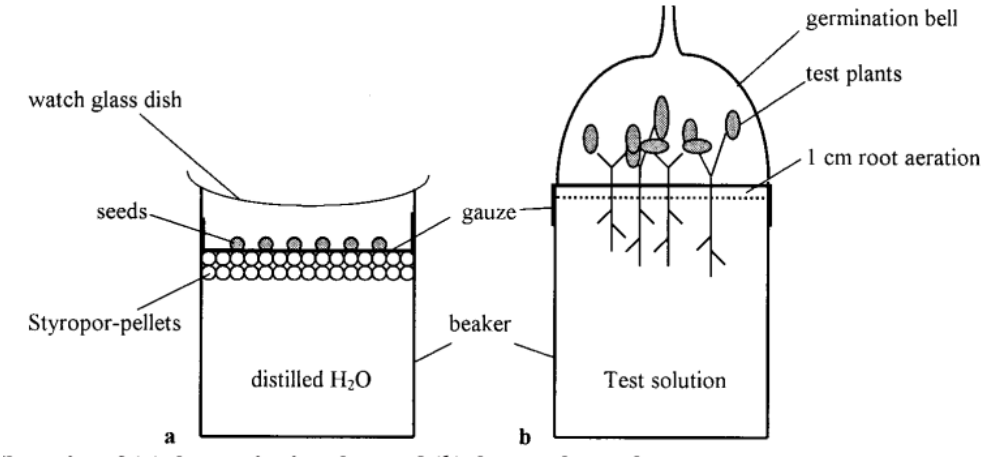

Figure 1.3: Seed germination using cotton gauze placed on styropor pellet. a, germination phase; b, growth test phase (Andersohn et al., 2002).

It is apparent that the use of additional substrates in toxicity testing with plants not only makes the tests laborious but also increases the cost of experiments especially when a large number of replicates are required. For example, Park, Yoon, et al. (2016) in an experiment compared the sensitivity of lettuce seeds to some toxicants on filter paper and six-well plate (direct exposure) using image analysis. He found that the toxicity of Hg and Cu to seeds germinated in well plates were several folds higher than those in the filter paper method.

With regard to bioassays in soil, Thomas and Thomas and Cline

(1985) modified Neubauer technique (an already existing technique for plant bioassay of soil) to simplify the procedures and reduce the cost. In this method, plastic Petri dishes with seeds were kept inside a plastic bag (Fig 1.4). This reduced the chance of daily watering and enabled periodic measurements easy (as the plastic bag can be opened periodically).

It seems from the ongoing discussion that the Petri plate or similar method (without support medium) gives the best results as it does not involve any interference of support material and requires no additional time for the preparation of support material. Moreover, the horizontal extension of roots, a problem encountered with Petri plate method (as discussed earlier), could be overcome by using image analysis tools to measure root length.

1.9 *Oryza sativa* in Toxicity Tests

Oryza sativa (rice), the most important staple food grain cultivated around the world, belongs to the family Poaceae (Gramineae). Rice has a history of more than 6000 years of being used a food crop (Huggan, 1995). It stands second to *Triticum aestivum* (wheat) with an annual production of 600 million tons (Delseny et al., 2001). According to a recent report, rice forms the staple food for approximately 3 billion people and constitutes about 80 % of their caloric consumption (Delseny et al., 2001). A recent statistics show that 37.5 % of global area of rice cultivation and 32 % of global rice production in the world belongs to Asia (Mohanty, 2014). India represents the country which has the largest rice area (43 million hectares) in the world (Mohanty, 2014).

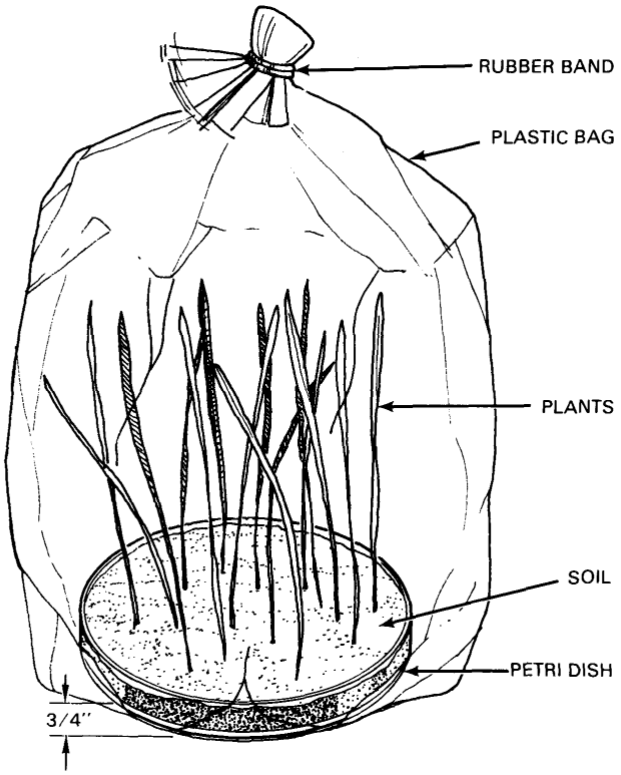

Figure 1.4: Modified Neubauer Technique for seed germination test with contaminated environmental samples (Thomas and Cline, 1985).

Besides being a valuable food crop, rice has the value as a tool in ecotoxicity testing for organic and inorganic contaminants. Rice is one of the species recommended by OECD for standard phytotoxicity tests (OECD, 2006). Nonetheless, *O. sativa* is underrepresented in ecotoxicity tests (Moore and Kröger, 2010). It is deplorable to observe that the potential of *O. sativa* as a tool in ecotoxicity tests has been neglected in the field of ecotoxicology. Rice, as a tool in ecotoxicity tests, has

several advantages over the traditional species. First, since it is a wetland and/or aquatic species (Correll and Correll, 1972), it is well suited for toxicity tests in aqueous media (Wang, 1993) such as effluents, municipal and domestic sewage, ambient water, and sediments. It is the unique ability of rice seeds to germinate anaerobically which makes it suitable for aqueous medium (He and Yang, 2013). Second, being an economically important crop, it satisfies the criteria to be used as a standard test species. Third, the roots produced by rice within 5 days of growth are generally shorter, which is a desirable feature in phytotoxicity tests as it excludes the possibility of seedling tangling resulting in handling difficulties (Wang, 1993). Fourth, unlike lettuce in which toxicant exposure leads to root decay, the rice produces stout roots when exposed to toxicants, thus simplifies handling (Wang, 1993). Moreover, the germination rate of rice is high when compared with some other species used in standard toxicity tests (Wang and Keturi, 1990). The longer shelf-life of rice facilitates its easy availability throughout the year (Wang and Keturi, 1990). Furthermore, as rice genome is fully sequenced (Yu et al., 2002), it is possible to include toxicogenomic endpoints in future studies (Brinke et al., 2015). All these features make this species an excellent choice for toxicity tests. Additionally, the availability of salt tolerant varieties of rice (Shylaraj et al., 2007) allows for its possible use in estuarine toxicity assessments. Rice has been employed to assess the toxicity of industrial as well as municipal effluents (Cordova Rosa et al., 2001; Rivera et al., 2013; Wang, 1990), and sediments (Brinke et al., 2015). In an earlier study, Nimmo et al. (2003) utilised *Zizania palustris* (wild rice), a close

relative of rice, to assess the toxicity of water collected from a creek.

Majority of toxicity studies on *O. sativa* are centred around heavy metals or other such compounds. Unlike *Lactuca*, *Myriophyllum*, and *Lemna* for which a number of studies using environmental samples are available, rice does not have sufficient toxicity data for environmental samples. Besides this, ecologically relevant endpoints such as NOEC (no observed effect concentration), LOEC (lowest observed effect concentration), and IC_x are rarely been reported for aquatic exposure of *O. sativa* compared to other species. A review of ECOTOX database of USEPA (2017) for *O. sativa* has shown that NOEL with 1467 entries followed by LOEL with 853 entries (which constitute terrestrial database) were the most widely reported estimates for phytotoxicity (Fig 1.5). Moreover, ecologically more relevant endpoint estimates such as IC_{10}, IC_{25} (or EC_{10} and EC_{25}) were not available in the aquatic database. It should be noted that estimates such as NOEC and LOEC are severely criticised by many authors due to its dependence on the test concentrations and lack of statistical plausibility (Festing, 2014; Fox, 2008; Hoekstra and Ewijk, 1993; Warne and Dam, 2008). The survey of Aquatic database showed that the maximum number of entries were made for NaCl (112 Records; Fig 1.6), followed by Sodium selenate (Na_2SeO_3; 78 records). The terrestrial database contained the highest number of entries for Copper chloride (CuCl; 312 records), followed by Fenoxaprop-P-ethyl (150 records), a widely used herbicide.

At present, standardised test methods are available for aquatic macrophytes such as *Lemna* and *Myriophyllum* only. An effort has been made

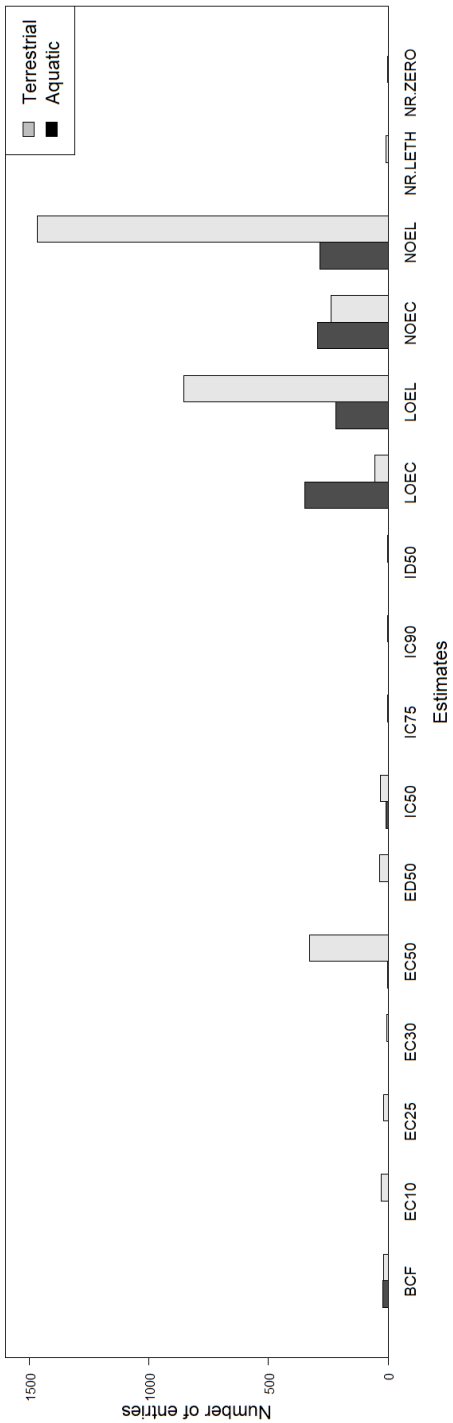

Figure 1.5: ECOTOX database for *O. sativa*. BCF = Bio concentration Factor; NR.LETH = 100%; NR.LETH = 100% mortality or 0% survival of organism; NR.ZERO = 0% mortality or 100% survival of organisms; NOEC(L)= no observable effect concentration (level); LOEC(L)= lowest observable effect concentration(level);EC, ED = Effective concentration or dose to x%; C, ID = Inhibition concentration or dose to x% of response.

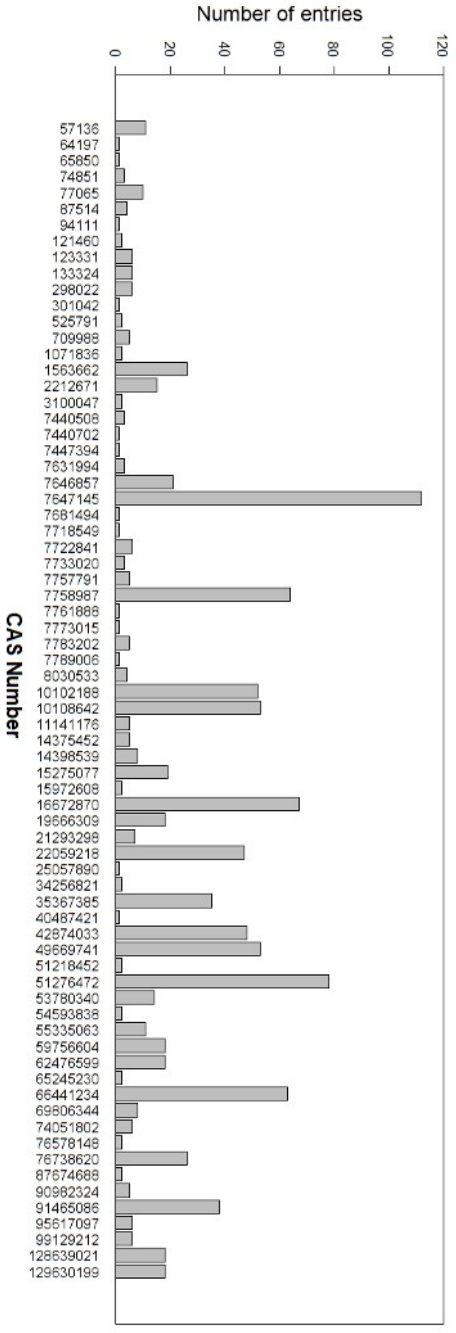

Figure 1.6: Chemicals for which concentration based toxicity data of *O. sativa* are available in Aquare (ECOTOX) database.

recently by Brinke et al. (2015) to develop a protocol for sediment-contact assay with *O. sativa*. These authors assessed the sensitivity of *O. sativa* to some selected toxicants in both spiked artificial sediment and natural sediment. They observed that both root and shoot were similarly sensitive to toxicants in spiked artificial sediments, whereas shoot was the only most sensitive organ in natural sediments. Above all, monocots like *O. sativa* has never been explored for the toxicity identification evaluation (TIE) - a protocol used to specifically identify the toxicants present in the contaminated samples, which is gaining attention in the field of ecotoxicology.

It is evident that *O. sativa* proves to be a promising choice in ecotoxicological investigations, and that further inquiries into the utility of this species as a tool in toxicity assessment are required to enrich the toxicity database.

Objectives of the present study:

- To generate phytotoxicity data of selected toxicants (cadmium, copper, lead, phenol, and sodium sodecyl sulfate) using *Oryza sativa*,
- To develop and validate Toxicity Identification Evaluation (TIE) protocol for liquid sample with *Oryza sativa*,
- To assess the utility of *Oryza sativa* for sediment toxicity tests, and
- To develop and validate sediment Toxicity Identification Evaluation (TIE) protocol with *Oryza sativa*.

Thesis is presented in six chapters:

- Chapter 1: General Introduction
- Chapter 2: Phytotoxicity of Selected Inorganic and Organic Compounds to *Oryza sativa*.
- Chapter 3: Toxicity Identification Evaluation (TIE) of a Chemical Mixture with *Oryza sativa*.
- Chapter 4: The Use of *Oryza sativa* in Sediment Toxicity Assessment of the River Periyar
- Chapter 5: Sediment Toxicity Identification Evaluation (TIE) of the River Periyar with *Oryza sativa*.
- Chapter 6: General Summary and Conclusion

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Chapter 2

Phytotoxicity of Selected Inorganic and Organic Compounds to *Oryza sativa*

2.1 Introduction

Excessive human population on the earth is causing a wide range of environmental changes which ultimately lead to environmental pollution. Changing land use patterns, unscientific agricultural practices, and industrial activities continue to pollute the land and water bodies at a global level. A wide variety of chemicals are released into the environment

during the human interaction with the environment. The agricultural practices release a wide array of agrochemicals including pesticides and heavy metals. The industrial effluents released into the water bodies generally contain chemicals in a quantity several folds higher than those found in the natural water bodies. The deadly cocktail of chemicals released by these industries usually contains inorganic (e.g. heavy metals) and organic (e.g. pesticides, phenols, surfactants) etc. Owing to their environmental relevance, some of these compounds have been used as reference toxicants in toxicity tests by some environmental agencies such as Environment Canada (EC, 1990) and EPA (USEPA, 2002a). Reference toxicants are the compounds used to compare the results of toxicity tests from different organisms (EC, 1990). Reference toxicants are also used to judge the comparability of test results from different laboratories. Additionally, they also enable us to judge the sensitivity and health of the test species. Inorganic toxicants such as cadmium ($CdCl_2$), hexavalent chromium, copper, sodium chloride, zinc, and organic toxicants such as phenol, 4-chlorophenol, sodium pentachlorophenate, sodium dodecyl sulfate (SDS) etc. are some of the commonly used reference toxicants.

A plethora of toxicity data for reference toxicants is available for animals. However, the availability of phytotoxicity data for reference toxicants still remains meagre. Some of the inorganic and organic contaminants including those used as reference toxicants and their effect on plants are discussed below.

2.1.1 Heavy Metals and Organics in the Environment

The term "heavy metal" is a loose term given to those metal elements and metalloids (semimetals) with relatively high density and atomic weight (Tchounwou et al., 2012). Heavy metals originate from several sources among which those involving geologic parent material are the important ones (Nagajyoti et al., 2010). Heavy metals such as Co, Cr, Mn, Ni, Cu, Zn, Cd, Hg, Sn, and Pb generally originate from geologic parent materials (Nagajyoti et al., 2010). Agricultural activities involving the use of fertilizers and pesticides contribute to a major portion of heavy metals (Cu, Hg, Pb, Cd, Mn, Zn, Cr, Co, Fe etc.) reaching the earth surface (Wuana and Okieimen, 2011). Industrial activities like mining and refinement processes release Cd, As and Fe. The diffuse source of metals is mainly represented by the chemical products used in our daily life (Zabel, 1993). Besides these, domestic effluents are another major source of heavy metals.

Heavy metal accumulation in the sediment is of special concern as sediments form the sink for several heavy metals and play a vital role in heavy metal transport and fate (Zhang et al., 2014). The uptake of heavy metals is controlled by several factors including pH, temperature, fertilizers, ORP (oxidation reduction potential), soil moisture, and plant energy supply to organs (Yamamoto and Kozlowski, 1987). In general high pH and low ORP reduce the metal availability (Misra and Mani, 1991), and thus cause a decrease in toxicity.

Acid volatile sulfide (AVS), the concentration of H_2S liberated by the addition of 1 N HCl, plays a major role in metal availability in sediments (Di Toro et al., 2005; Meysman and Middelburg, 2005). Sulphides of Fe and Mn represent the important fraction of AVS-metal complex in the sediment (Zhang et al., 2014). As Fe and Mn are more soluble than cationic metals, the latter can displace the Fe or Mn from AVS-metal complex to form less soluble metal sulfide (USEPA, 2007). Fe and Mn oxides generally precipitate at the more oxygenated sediment surface layers, which subsequently lead to reduced metals mobility.

Sediment organic matter (SOM) is another important factor that contributes to the metal availability in sediments (Di Toro et al., 2005; Selck et al., 1999). SOM comprises a heterogeneous pool of substances originated diagenetically through microbial activity (Hong et al., 2010). Heavy metal ions can bind to SOM to form complexes as depicted in the Fig. 2.1 leading to reduced metal availability and ultimately reduced toxicity.

Cadmium

The heavy metal cadmium (Cd) belongs to group 12 with an atomic number 48 and melting point 767°C. In nature, Cd is present in the ores of Zn, Cu, and Pb (Sarkar et al., 2013). Industries that produce solders, colourants, plastic stabilisers, cadmium rods, electroplated materials, and nickel-Cd batteries are the major industrial sources of Cd (Sanità di Toppi and Gabbrielli, 1999; Sarkar et al., 2013). Plants generally express Cd toxicity as stunting, leaf rolls and chlorosis (Benavides et al., 2005). The

Cd toxicity operates mainly through structural destruction or inactivation of proteins by Cd binding to sulphydryl groups (Van Assche and Clijsters, 1990), formation of free radicals and reactive oxygen species (Fornazier et al., 2002; Sandalio et al., 2001).

However, there are several plant species which have evolved metal tolerance mechanisms (Prasad, 1995) to reduce the Cd toxicity mainly by depressing Cd bioavailability (Dong et al., 2007). Since Cd shows relatively low phytotoxic potential (Brinke et al., 2015), the toxic symptoms it produces usually remain externally unobservable and hence it easily reaches the humans. Thus the consumption of Cd tolerant food crops poses a serious health risk to human population and therefore, Cd requires a special attention with regard to phytotoxicity.

Copper

Copper (Cu) is one of the metals which do not require extraction from ores. As a member of group 11, it has an atomic number 29 and a melting point 1084.62°C. Copper exists naturally, but its concentration on the earth surface is increasing due to the excessive use of fungicides, herbicides, and organic fertilizers derived from sludge and manure (Panou-Filotheou, 2001). Since copper is an essential element, its deficiency or excess may affect the normal growth and development of plants (Yruela, 2005). Cu forms the structural component of regulatory proteins and partakes in mitochondrial respiration, oxidative stress responses, photosynthesis, hormone signalling and cell wall formation (Puig, 2014; Raven et al., 1999; Yruela, 2005). As a metal with redox activity, copper enhances the

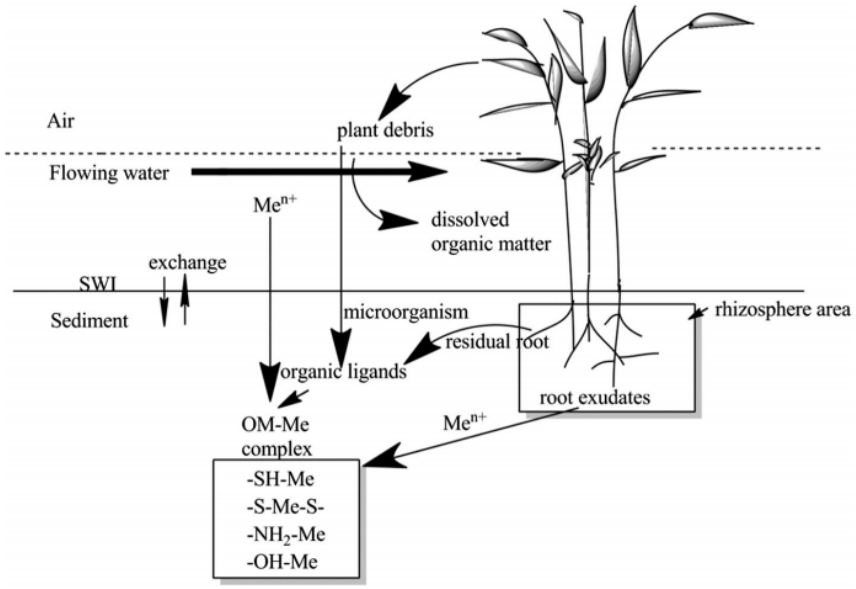


Figure 2.1: Relationship between sediment organic matter (SOM) and metal ions (Zhang et al., 2014).

formation of toxic hydroxyl radicals, which subsequently leads to damage of DNA and other cell components (Halliwell and Gutteridge, 1984). The symptoms of copper toxicity in plants include chlorosis, arrested growth, wilting, desiccation, damage of root cuticle, and reduced root hair production (Kuhns and Sydnor, 1976; Maksymiec, 1998; Sheldon and Menzies, 2005).

Lead

Lead (Pb; atomic number 82; melting point 327.46°C) comes under group 14 in the periodic table and is one of the most important toxic metals of concern. This metal is rarely found in free form and it usually forms a variety of molecules by combining with other elements. Though Pb

exists on the earth along with other metals, its main contributors in the environment are paints, batteries, fossil fuels, coolants, ammunition, glasses, agrochemicals, solid wastes and effluents (Pinho and Ladeiro, 2012).

Since Pb is a non-essential element, plants lack channels for Pb transport. The exact mechanism of Pb transport into the root cells still remains to be well understood (Pinho and Ladeiro, 2012). Though it has already been proven that the uptake of Pb is generally restricted to root, still there exist some controversies regarding this fact (Lane and Martin, 1977; Miller and Koeppe, 1971). A higher quantity of Pb is observed in dicot roots than monocot roots (Huang and Cunningham, 1996). Generally, the undesirable amounts of Pb in plants act by disrupting the tonoplast and plasmalemma, inhibiting the enzymes, mineral nutrition, water relations, photosynthesis, and hormonal activities (Sharma and Dubey, 2005). The ability of Pb to interfere with the carrier proteins is the main reason for the inhibition of mineral nutrition due to Pb toxicity (Xiong, 1997). As with the Cd, the plant tolerance to Pb creates a serious problem of it reaching humans and other animals through feedings.

Phenol

Phenol (C_6H_5OH), otherwise called carbolic acid or Benzol, is a weakly acidic, volatile compound that comprises a phenyl and a hydroxyl group. The phenol forms phenolate ion ($C_6H_5O^-$) at high pH and its pKa is 10 (Kromidas, 2008). Phenol belongs to one of the first compounds among the priority pollutants list of the US Environmental Protection Agency

(Michalowicz and Duda, 2007). Phenol is derived from petroleum and is mainly used in plastic, pharmaceutical, textile, and pesticide industries. Several useful compounds used in our daily life are synthesised from phenol.

A vast number of phenolic compounds in the form of secondary metabolites are produced by plants themselves for cellular defence. However, such phenolic compounds have also been shown to be toxic to plants (Shabala, 2011). The phenolic compounds found in plants are phenolic derivatives with diverse structure and their concentration generally ranges from 100 to 500 mg/Kg dry matter (Glass, 1973; Wu et al., 2001).

Pure phenol is more toxic than its derivatives (Todorović, 2003). Phenolic compounds are organic acids with high toxicity at lower pH and they undergo disintegration as the pH increases (Armstrong and Armstrong, 2001; Drew and Lynch, 1980; Shabala, 2011).

Phenol toxicity depends on its hydrophobicity and the placement of its substituents in the molecule (Michalowicz and Duda, 2007). Phenolic compounds cause toxicity by affecting the cell organelles like ER, mitochondrion, and nucleus. It also causes mutagenesis by interfering with DNA. Due to its popularity, phenol is used as a model substance in toxicity studies (Schie and Young, 2000).

Sodium Dodecyl Sulfate (SDS)

Sodium dodecyl sulfate (SDS), an organosulfate compound, also known as sodium lauryl sulfate (SLS), is an anionic surfactant with a sulfate group and a long tail of 12 carbon atoms. It has the formula $CH_3(CH_2)_{11}SO_4Na$.

Its hydrophilic head and hydrocarbon tail give it detergent-like properties. Anionics, the earliest surfactants used by mankind, are now widely used in research activities, and industries that produce pharmaceutical, biotechnological, and agricultural products (Cserháti et al., 2002; Liwarska-Bizukojc et al., 2005). According to a report, the consumption of detergent and softener products in European countries has reached a value of 4250000 and 1190000 tons/year, respectively (Pettersson et al., 2000). Surfactants, due to their compositional diversity, and widespread use, have gained much interest in research field (Liwarska-Bizukojc et al., 2005).

Surfactants can be classified into three types, anionic, non-ionic, and cationic, which include a variety of compounds among which Linear alkylbenzene sulphonates (LAS), alkylphenol ethoxylates (APE), alkyl ethoxy sulfates (AES), alkylethoxylates (AE), alkyl sulfates (AS), and quaternary ammonium compounds (QAC) are the commercially important ones (Ying, 2006). Anionic and non-ionic surfactants can cause toxicity at concentrations as low as 0.0025 and 0.3 mg/L, respectively (Pettersson et al., 2000). Despite all their benefits, surfactants like SDS cause toxicity which is operated through diverse pathways (Li, 2008). The toxicity due to surfactants (including SDS) is mainly because of their binding with proteins and subsequent modification of enzymes and cell organelles (Cserháti et al., 2002). It should however, be noted that no sufficient data regarding the surfactants are available at present to make any detailed understanding of their action in biological system (Cserháti et al., 2002).

Studies on lower plants such as algae have demonstrated variable effects of SDS. The effect of SDS on higher plants such as *Sinapsis*

alba was found to be stimulatory (10 mg/L) at 72-h (Ostroumov, 2005). The phytotoxicity data of SDS for many vascular plants still remains unavailable and hence, it needs to be well documented.

2.1.2 Combined Effect of Toxicants

Most studies in ecotoxicology are centred on the effect of toxicants individually. In fact, most chemicals in the environment co-occur with others, and therefore, results from single-substance bioassay may not easily be applied to environmental risk assessment. There are two approaches in the field of ecotoxicology with regard to mixture toxicity. The first one ('bottom-up approach') depends on the mode of action or site of action of components of mixture for model selection, whereas the second one ('top-bottom approach') depends on toxicity rather than component interaction (Warne, 2003).

The joint action of compounds can be classified into three; additivity, synergism and antagonism. The effect can be said to be additive when the mixture toxicity is equal to the sum of toxicity of individual components in the mixture. Synergism occurs when the mixture toxicity exceeds the sum of toxicity of individual components, whereas antagonism occurs when the mixture toxicity is less than the sum of toxicity of individual components (Calabrese, 1990; Calamari and Alabaster, 1980; Marking, 1985; Marking and Dawson, 1975). The joint action of chemicals in the mixture can be of different types as follows (Bliss, 1939; Hewlett and Plackett, 1952): In 'simple similar joint action' (concentration addition), the toxicants act on the same site but does not interfere with the biological activity of one

another. In 'independent joint action' (response addition), the chemicals act on different sites and they also do not affect the biological activity of one another. The 'complex similar and dependent joint action' involves interference of at least one compound in the mixture with the biological activity of at least one other compound in the mixture.

At present, there is a gap of sufficient knowledge regarding the toxicity of chemical mixtures to vascular plants. Many of the studies on combined effects of chemicals come from the field of herbicide toxicity. Regarding mixture toxicity to plants, a recent work has shown that herbicide mixtures (binary) atrazine/simazine, atrazine/metolachlor and atrazine/terbuthylazine were synergistic to microalga *Pseudokirchneriella subcapitata* (Pérez et al., 2011). Another study with *Pseudokirchneriella subcapitata* and *Lemna* has shown that herbicide mixture could also result in absence of synergism (Munkegaard et al., 2008). Combined effect of arsenic and cadmium on Triticum aestivum was found to be synergistic in solution, but antagonistic in soil. In *O. sativa*, the accumulation of metals due to exposure of mixture containing cadmium and copper was shown to be dependent on the genotype of rice (Huang, Hu, et al., 2009).

2.1.3 Effect Concentrations and NOEC in Toxicity Test

The numerical data generated in ecotoxicology generally include point estimates such as EC_x, IC_x, and LC_x, or hypothesis test based NOEC and LOEC (USEPA, 2002b; Warne and Dam, 2008). EC_x, IC_x, and LC_x represent the concentration that causes an x percentage (e.g. 5,

10, 20, 25%) of effect, inhibition, and lethality, respectively. LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) represent the lowest concentration that differs significantly from control and the highest concentration that does not differ significantly from control, respectively (OECD, 2011; Warne and Dam, 2008).

Generally, in ecotoxicology, IC_{50} is used to compare the toxicity of different xenobiotics. Even though IC_{50} provides a more reliable and precise values with narrow confidence intervals (CI), it is not a biologically safe concentration to be used in environmental regulations. Hence, IC_{50}s are generally used to compare the toxicity of xenobiotics rather than to make regulatory decisions. Most regulatory agencies use NOEC as a protective estimate in environmental decision making as it represents much safer concentration. However, NOECs has received many criticisms in that it is merely based on statistical significance rather than on biological significance (Fox, 2008; Hoekstra and Ewijk, 1993; Kooijman, 1996; Warne and Dam, 2008). Critics also argue that the NOEC depends on the concentration chosen for the toxicity test and may not provide realistic value. Unlike in the case of IC_x estimates, confidence intervals cannot be computed from NOEC. Considering these issues, USEPA (2002b) suggested low percent-effect point estimate (IC_p) such as IC_{25}. Recent studies also support the use of IC_{25} as an alternative to NOECs (Moore et al., 2000; Oliveira-Filho et al., 2008). EPA uses linear interpolation, a non-parametric method, to calculate IC_ps. Although it does not require any model assumptions and is easy to compute, the method of IC_p estimation has also been questioned as it smoothes the data during its

computation, which results in biases. Most recent guidelines by some regulatory agencies such as Environment Canada recommends nonlinear regression for the computation of point estimates (e.g. IC_{25}), and restrict the use of linear interpolation method as a last resort only (EC, 2005).

In fact, the controversy regarding whether to use NOEC or IC_x still continues and it calls for further research in this regard. Although IC_ps are biologically relevant endpoint estimates, they have rarely been compared with LOECs and NOECs in chronic toxicity tests (Marchini et al., 1992). The validity of NOEC and EC_x can usually be evaluated by comparing them with estimates derived from species sensitivity distributions (SSD) - the models describing variation in sensitivity of species to toxicants (Posthuma et al., 2002). For example, in a recent study, (Iwasaki et al., 2015) compared the NOEC and 10% effect concentration to assess their impact on HC_5s (the concentrations which affect 5% of the species) and found that NOEC or point estimate for low effect (EC_x) does not influence the HC_5s if used carefully.

It seems from the ongoing studies, that a reasonable approach would be to report both NOECs and ICs (e.g. IC_{25}). Furthermore, despite being widely criticised, NOECs are still recommended by the Organization of Economic Cooperation and Development (OECD) and the United States Environmental Protection Agency (USEPA) to be reported along with the IC values (OECD, 2011; USEPA, 2002a,b). The problem associated with uncertainties in the lack of confidence intervals of NOECs can be avoided by using simultaneous confidence intervals from relative effects. The solution comes from bioequivalence studies in which ratios of means

of control and treatments are compared to compute confidence intervals (Delignette-Muller et al., 2011).

Traditional methods of NOEC calculation involves multiple comparisons between a control and several treatments, which make it extremely difficult to estimate confidence intervals from it. However, recent advancements in computational toxicology have made it easier to compute simultaneous confidence intervals for relative effects between a control and multiple treatments. Such methods have been implemented in R packages like mratios (Delignette-Muller et al., 2011; Dilba et al., 2012). Simultaneous confidence intervals can be used to infer the bounds of uncertainties (BOU) from IC_x, EC_x, and/or LC_x estimates and thus the gap between EC_x estimates and NOEC can be filled (Delignette-Muller et al., 2011).

Though a lot of reports regarding the toxicity of inorganic and organic toxicants to *O. sativa* exist, many of them are studies involving limited concentration ranges which make it difficult to calculate IC values. Besides this, the utilisation of *O. sativa* in Ecotoxicology is less explored. Only a few reports concerning inhibition concentration of toxicants to *O. sativa* exist in the open literature. Therefore, the present study aims to generate toxicity data of some inorganic and organic toxicants with special reference to some commonly used reference toxicants.

2.1.4 Endpoints in Toxicity Tests

Though several advancements including molecular techniques exist in ecotoxicology today, classical endpoints involving morphometric parameters

still play a relevant role in risk assessment as they are cost effective and easy to measure. Moreover, classical endpoints such as seed germination, root and shoot elongation have been found to be more sensitive than biochemical endpoints for some compounds (Pei-jun et al., 2005). The differential response of shoot and root to the same toxicant can be useful in the detection and quantification of toxicants as this kind of response is compound specific. Though low effect percentage is claimed protective, it would not be a wise decision to choose it as the confidence limit is wide.

The aim of the present study was to generate phytotoxicity data of selected inorganic and organic contaminants with special reference to reference toxicants commonly used in toxicity tests.

2.2 Materials and Methods

2.2.1 Test Chemicals

Three heavy metals ($CdCl_2$, $CuSO_4$, and $Pb(NO_3)_2$) representing inorganic toxicants and two organic toxicants (phenol and sodium dodecyl sulfate) were used in the toxicity test. All the reagents used were analytical grade.

2.2.2 Test Species

Oryza sativa var. Jyothi was selected for the study. Rice is also a test species recommended by OECD. The rice seeds were obtained from Regional Agricultural Research Station, Pattamby, Palakkad, Kerala, India. The collected seeds were placed in a glass bottle (70 × 20 cm) with rubber lid and kept in the refrigerator (40°C) until use.

2.2.3 Toxicity tests

Individual Toxicant Tests

Toxicity tests involved both individual and combined exposures of toxicants. For individual exposures, a preliminary range finding test was performed to decide the concentration ranges to be used in definitive tests. For definitive tests, the ranges of toxicant concentrations selected were 0, 3.75, 7.5, 15, 30, 60, and 120 mg/L for $CdCl_2$, 0, 1, 2, 4, 8, 16, and 32 mg/L for $CuSO_4$, 0, 6.25, 12.5, 25, 50, 100, and 200 mg/L for $PbN(O_3)_2$, 0, 31.25, 62.5, 125, 250, 500, and 1000 mg/L for both phenol and sodium dodecyl sulfate. Distilled water was used for preparing the dilution series (Park, Yoon, et al., 2016). The seeds were screened for abnormal and damaged ones and were removed if present. Bioassays were performed in triplicates of glass Petri plates (90 × 15 mm) with 10 ml test solution and 10 seeds each. Seeds were directly placed in test solution without any additional supporting material. The seeds were incubated at 28±2°C under cool white light (300 µmol/m^2/s, 14:10 light:dark) for 4 days (96-h). The germinating seeds were photographed at 72 and 96-h (test termination) using a digital camera (Kodak EasyShare M531). Seeds were considered germinated when the radicle protruded out at least 1 mm. The endpoints studied were root length, shoot length, seedling length, and seed germination. Root, shoot, and seedling lengths were measured using image analysis software Fiji (Schindelin et al., 2012). IC_{50} and IC_{25} were calculated for each compound and endpoints selected.

Mixture Toxicity Test

For combined effects test, a binary mixture of Cd and phenol each at their equitoxic concentrations of 5X, 2.5X, 1.25X, 0.625X, 0.3125X, and 0X IC_{50} of root were used in exposure. The experimental conditions were the same as those used for individual exposures. The combined effects were evaluated using toxic unit (TU) and mixture toxicity index (MTI) approaches. For individual toxicant, TU (Eq. 2.1, 2.2) is the reciprocal of toxicant concentration that causes 50% (or x%) of inhibition (or other response of concern) relative to control (USEPA, 1991; Zeb et al., 2016). The sum of toxic unit (Eq. 2.3) is used to predict the toxicity of mixture.

$$TU_i = \frac{c_i}{IC_{50,i}} \tag{2.1}$$

where C_i, $IC_{50,i}$, and TU_i represents the actual concentration, inhibition concentration (50%), and toxic unit, for ith compound.

$$TU_{mix} = \sum_{i=1}^{n} TU_i = TU_{Cd} + TU_{phenol} \tag{2.2}$$

where TU_{mix} represents the toxic unit calculated for the mixture, and n is the total number of toxicants.

Assuming additivity, a binary mixture containing a concentration of both the compounds at 0.5 X IC_{50} would result in 1 X IC_{50} (Groten et al., 2001; Marking and Dawson, 1975). In other words, IC_{50mix} (IC_{50} for mixture) = 1 TU represents additivity. Similarly, $IC_{50mix} > 1$ TU and $IC_{50mix} < 1$ TU represents greater (synergistic) than and less than (antagonistic) additive actions, respectively.

Additivity index was calculated in accordance withMarking and Dawson (1975), in which S denotes sum of the toxicity of compound A and B (Eq. 2.3). The IC_{50} of the individual compound and mixture is represented by i and m, respectively. Additivity index can be computed from the S as shown in Eq. 2.4. The sign of AI value conveys the synergistic or antagonistic action of the chemical mixture.

$$S = \frac{A_m}{A_i} + \frac{B_m}{B_i} \tag{2.3}$$

$$AI = \frac{1}{S} - 1(S \leqslant 1) \; or \; AI = (S - 1) + 1(S \geqslant 1) \tag{2.4}$$

MTI, proposed by Könemann (1981) is calculated as per the Eq. 2.5:

$$MTI = \left(\frac{logM}{logn}\right) \tag{2.5}$$

where M is the sum of incipient IC_{50} for each toxicant (Eq. 2.2), and n is the total number of toxicants in the mixture.

The MTI values represent antagonism (MTI $<$ 0), no addition (MTI $=$ 0), partial addition (0 $<$ MTI $<$ 1), concentration addition (MTI $=$ 1), and supra addition (MTI $>$ 1).

AI or MTI were not computed for responses other than root elongation as the concentrations of Cd and phenol used were not equitoxic to those responses.

2.2.4 Data Analysis

Point estimates (IC_{50}, LC_{50}, IC_{25}, and LC_{25}) were computed using drc package (Ritz et al., 2015) which runs under R (version 3.4.1) environment

(R Core Team, 2017). The computations were performed using nonlinear regression method. When more than one highest concentration produced zero response, all those concentrations except the lowest one were excluded from the calculation of point estimates. Data were Box-Cox transformed whenever the distribution assumptions were found violated (Ritz et al., 2015). NOECs were derived by multiple comparisons (one-tailed) between control and treatments using multcomp package (Hothorn et al., 2008). Bounds of uncertainties (BOU) were computed (for both IC/LC_{25} and IC/LC_{50}) using mratios package (Delignette-Muller et al., 2011; Dilba et al., 2012).

2.3 Results

2.3.1 Individual Toxicant Tests

Toxicity of $CdCl_2$

A significant difference in IC_{25} values of root and shoot were observable only after 96 hours (Table 2.1, 2.2; Fig. 2.2a, 2.2b). The IC_{25} values of seedling (5.38 and 3.78 mg/L at 72-h and 96-h, respectively) and root (4.83 and 3.01 mg/L at 72-h and 96-h, respectively) did not differ significantly even after 96 hours of exposure. The sensitivity of root (based on IC_{50}) to $CdCl_2$ at 72 and 96 hours differed by a factor of 1.5. Extending the test duration to 96-h did not bring any significant difference in the IC_{50} value of shoot length when compared with that of 72-h. The 72-h IC_{50} for root growth was 7.61 mg/L, which further reduced to 5.05mg/L at 96-h. While the LC_{50} for seed germination increased from 15.0 to

18.6 mg/L at 96-h, the IC_{50} for shoot growth decreased (non-significant, $p > 0.05$) from 29.5 mg/L to 21.5 mg/L. The difference between the LC_{50} germination and IC_{50} root was approximately 1.9 and 3.6-folds at 72-h and 96-h, respectively. However, the ratio between IC_{50} values was more or less similar at different time points (3.8 and 4.2 at 72-h and 96-h, respectively) when root and shoot were compared. The seed germination was completely inhibited at 30 mg/L and 60 mg/L for 72-h and 96-h tests, respectively (Fig. 2.2c). However, the shoot growth was not completely inhibited even at 120 mg/L for any of the time points studied.

The IC_{50} of root at 96-h and IC_{25} of root and seedling at 72-h were closer to the 72-h IC_{50} of *S. alba* reported by Fargašová (2004). In the case of seedling length at 72-h and germination at 96-h, the NOEC (Table. 2.10) values ($<$ 3.75 mg/L and 7.5 mg/L, respectively) fell below the lower bounds of IC_{25} (4.3 - 6.5 mg/L, and 13 – 18 mg/L, respectively). Of all the toxicants tested, Cd produced the lowest (1.21 for shoot length at 72-h) and highest (17.47 for germination at 72-h) values for the slope parameter.

Toxicity of $CuSO_4$

None of the $CuSO_4$ concentration ranges used was sufficient enough to reduce the shoot growth even at 25% effect level (Table 2.4; Fig. 2.3a, 2.3b). At 96-h, the shoot demonstrated a significant hormetic response ($f = 10.2$, $p < 0.01$) between the concentration ranges 0.5 and 8 mg/L (mean shoot length = 12.2 mm and 9.2 mm, respectively, compared to 7.7 mm at control). Though root and seedling showed a hormetic tendency,

Table 2.1: IC_{50} (LC_{50} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to $CdCl_2$. BOU = bounds of uncertainty (Delignette-Muller et al., 2011).

Duration	Endpoint	LC/IC_{50}	95% CI		BOU (50%)	
			Lower	Upper	Lower	Upper
96-h	Root	5.05	4.3	5.8	3.7	7.5
	Shoot	21.5	16	27	7.5	60
	Seedling	8.14	6.8	9.5	3.75	15
	Germination	18.6	16	21	15	30
72-h	Root	7.61	6.1	9.1	3.75	15
	Shoot	29.5	13	46	7.5	> 120
	Seedling	9.66	8.3	11	3.75	15
	Germination	15	14	16	7.5	30

Table 2.2: IC_{25} (LC_{25} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to $CdCl_2$. BOU = bounds of uncertainty (Delignette-Muller et al., 2011)

Duration	Endpoint	LC/IC_{25}	95% CI		BOU (50%)	
			Lower	Upper	Lower	Upper
96-h	Root	3.01	2.3	3.7	0	7.5
	Shoot	12.1	7.5	17	0	30
	Seedling	3.78	2.8	4.7	0	7.5
	Germination	15.4	13	18	7.5	30
72-h	Root	4.83	3.5	6.2	0	7.5
	Shoot	11.9	2.5	21	0	120
	Seedling	5.38	4.3	6.5	3.75	7.5
	Germination	14.1	6.2	22	7.5	15

the responses were not significant ($p > 0.05$). The result suggests longer than 96-h exposure to achieve a 50% shoot growth inhibition at the tested concentration ranges. The root (IC_{50}= 6.48 mg/L) was 3.7 times as sensitive as seed germination (LC_{50} = 24.1 mg/L) at 96 hours.

The 72-h exposure was insufficient to produce a significant difference between IC values of root and seedling at 25 and 50% effect levels (Table 2.3). The seed germination continued in all the concentration ranges tested for both the time points with 100% germination recorded at 8 mg/L (Fig. 2.3c). At the topmost concentration (32 mg/L), the germina-

tion was reduced to 47.4 and 25.5% for 72-h and 96-h, respectively. At 72-h, the LC_{50} for seed germination (30 mg/L) was closer to the highest concentration tested. No significant difference in LC_{25} ($p > 0.05$) was observed for the of seed germination at both the test durations (72-h LC_{25} = 20.6 mg/L; 96-h LC_{25} = 18.4mg/L). The root IC_{50} for two test durations differed by a factor of 1.7. No difference in calculated IC_{50} (or LC_{50}) was observable among root at 96-h and seedling and germination at 72-h. The 72-h NOEC (8 mg/L) for root (Table. 2.10) was closer to the corresponding IC_{25}. Similar observation was obtained for germination IC_{25} at 96-h (NOEC = 16 mg/L; IC_{25} = 18.4 mg/L).

Toxicity of $PbN(O_3)_2$

The results indicated that *O. sativa* could tolerate the toxicity of lead at lower ranges of exposure. The shoot was more tolerant than root to $PbN(O_3)_2$ (Fig. 2.4a, 2.4b). The 72-h IC_{50} for shoot was greater than the topmost concentration (200 mg/L) used (Table 2.5). There was no significant difference ($p > 0.05$) between 72-h and 96-h IC values of root, shoot, and seedling, indicating a delay in eliciting the toxic response from the plant. The 96-h IC_{50} was 43.7 and 182.0 mg/L for root and shoot, respectively.

Though there was a complete inhibition of seed germination at 200 mg/L $PbCl_2$ in 72-h incubation (Fig. 2.4c), extending the incubation to 96-h resulted in mean germination percentage of 25% (% of control). Though the shoot IC_{50} did not show a significant difference between the exposure periods, the shoot IC_{25} at 72-h showed a significant reduction by

Table 2.3: IC_{50} (LC_{50} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to $CuSO_4$. BOU = bounds of uncertainty (Delignette-Muller et al., 2011)

Duration	Endpoint	LC/IC_{50}	95% CI		BOU (50%)	
			Lower	Upper	Lower	Upper
96-h	Root	6.48	4.8	8.1	2	16
	Shoot	> 32	19	91	32	> 32
	Seedling	13.6	9.8	17	8	> 32
	Germination	24.1	20	28	16	> 32
72-h	Root	10.7	7.9	13.5	4	> 32
	Shoot	> 32	–	–	0	> 32
	Seedling	12.7	7.8	17.5	4	> 32
	Germination	30.06	22.8	37.3	50	200

a factor of 2 (LC_{50} = 192.9 and 96.1 mg/L for 72-hand 96-h, respectively). Except for seed germination (LC_{50} = 93.8 and 120.7 mg/L for 72-hand 96-h, respectively), no other parameters demonstrated a significant change in IC_{50} across the exposure periods. It should be noted that root IC_{50} values for Cd and Cu were significantly different at 72-h ($p < 0.05$), but it became non-significant ($p > 0.05$) as the exposure duration increased. Pb elicited a tendency of hormesis in shoot at 96-h (f = 1.36, $p > 0.05$).

NOEC (25 mg/L) for seedling at 96-h was slightly lower than the confidence interval (CI = 26 - 45) of IC_{25} (Table 2.6, 2.10). Similarly,

Table 2.4: IC_{25} (LC_{25} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to $CuSO_4$. BOU = bounds of uncertainty (Delignette-Muller et al., 2011).

Duration	Endpoint	LC/IC_{25}	95% CI		BOU (25%)	
			Lower	Upper	Lower	Upper
96-h	Root	4.5	2.9	6.1	1	8
	Shoot	> 32	19	57	16	> 32
	Seedling	7.25	4.8	9.7	2	16
	Germination	18.4	15	22	8	32
72-h	Root	8.06	5.6	11	0	16
	Shoot	> 32	–	–	0	> 32
	Seedling	8.01	5	11	0	32
	Germination	20.6	15	26	0	> 32

NOEC (50 mg/L) for germination both at 72-h and 96-h was lower than the CI (55 -85 and 65 -99) of IC_{25}.

Toxicity of Phenol

The inhibitory effect of phenol on root was high at first, but gradually ceased as the exposure time increased (Fig. 2.5a, 2.5b). This was evidenced by greater IC (or LC) values for all the growth parameters at 96-h compared with those at 72-h (Table 2.7, 2.8). Between 72 and 96 hours, there was a reduction in IC_{50} by 2, 1.8 and 1.3-folds and in IC_{25} by

Table 2.5: IC_{50} (LC_{50} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to $PbN(O_3)_2$. BOU = bounds of uncertainty (Delignette-Muller et al., 2011).

Duration	Endpoint	LC/IC_{50}	95% CI		BOU (50%)	
			Lower	Upper	Lower	Upper
96-h	Root	43.7	31	56.2	12.5	100
	Shoot	182	101	263	12.5	> 200
	Seedling	61.5	50	73	25	100
	Germination	120.7	98	143	50	200
72-h	Root	49.7	40	59	25	100
	Shoot	>200	158	331	100	> 200
	Seedling	70.7	55	86	25	200
	Germination	93.8	80	108	50	200

3, 2.4 and 1.6-folds in the case of seedling, root, and shoot, respectively. The IC_{50} values of root and shoot differed by a factor of 3.7 and 2.6 at 72-hand 96-h, respectively. There was a 6-fold difference between IC_{25} values of root and shoot at 72-h. After 96 hours of incubation, the percentage of germinated seeds at 500 mg/L increased from 36.6 to 70 (Fig. 2.5c). All the seeds lost the ability to produce root and shoot at 1000 mg/L. Seed germination was not much affected by the change in incubation period as evidenced by closer IC values at both time points. For phenol, all the endpoints (root, shoot, and seedling) at 96-h had LCI

Table 2.6: IC_{25} (LC_{25} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to $PbN(O_3)_2$. BOU = bounds of uncertainty (Delignette-Muller et al., 2011).

Duration	Endpoint	LC/IC_{25}	95% CI		BOU (25%)	
			Lower	Upper	Lower	Upper
96-h	Root	29.1	15	43	0	100
	Shoot	96.1	58	135	12.5	> 200
	Seedling	35.3	26	45	12.5	100
	Germination	81.8	65	99	50	200
72-h	Root	35.6	24	47	0	100
	Shoot	192.9	165	221	100	> 200
	Seedling	38.5	25	52	0	100
	Germination	70.1	55	85	25	100

of IC_{25} greater than their calculated NOECs (Table 2.10).

Toxicity of SDS

The effect of SDS on growth of the plant was much slower as it could not produce a significant difference ($p > 0.05$) in IC values between 72 and 96 hours for any morphological endpoint (Table 2.9). However, a significant difference between IC values of root and shoot was observed at 25% and 50% effect levels (Table 2.9). The root demonstrated a statistically significant ($p < 0.05$) hormetic effect (11.86 to 20.96% of control) from

Table 2.7: IC_{50} (LC_{50} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to phenol. BOU = bounds of uncertainty (Delignette-Muller et al., 2011).

Duration	Endpoint	LC/IC_{50}	95% CI		BOU (50%)	
			Lower	Upper	Lower	Upper
96-h	Root	186.2	155	217	125	500
	Shoot	487.5	477	498	250	1000
	Seedling	301.5	251	352	125	500
	Germination	526.6	365	688	500	1000
72-h	Root	103.1	57.5	149	31.25	500
	Shoot	381.5	293	470	125	500
	Seedling	151.3	74.3	228	31.25	500
	Germination	481.2	407	556	250	1000

31.25 to 62.5 mg/L SDS both at 72-h and 96-h exposures (Fig. 2.6a, 2.6b); this effect was reflected especially in lateral root induction at these concentration ranges. The calculated IC_{25}s did not match with the NOECs (Table 2.11) in the case of root both at 72 and 96-hrs as the LCI were well above the corresponding NOEC values. The bound of uncertainty for germination at 96-h covered the entire ranges of tested concentrations (Table 2.9; Fig. 2.6c).

Based on IC (or LC) vales, the toxicants tested can be arranged in the descending order of toxicity as shown in the tables (Table 2.12, 2.13).

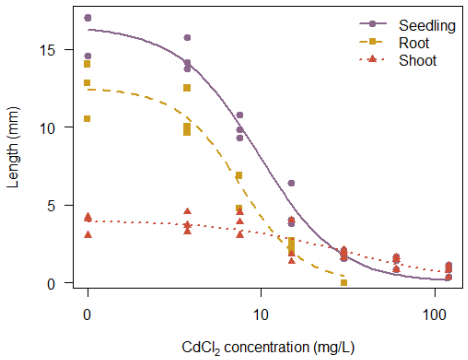
Table 2.8: IC_{25} (LC_{25} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to phenol. BOU = bounds of uncertainty (Delignette-Muller et al., 2011).

Duration	Endpoint	LC/IC_{25}	95% CI		BOU (25%)	
			Lower	Upper	Lower	Upper
96-h	Root	111.7	83.2	140	31.25	250
	Shoot	451	433	469	0	500
	Seedling	200	146	254	0	500
	Germination	492.6	443	542	250	500
72-h	Root	46.1	15	77	0	250
	Shoot	281.2	154	409	0	500
	Seedling	66.3	12	120	0	250
	Germination	435.1	206	664	250	500

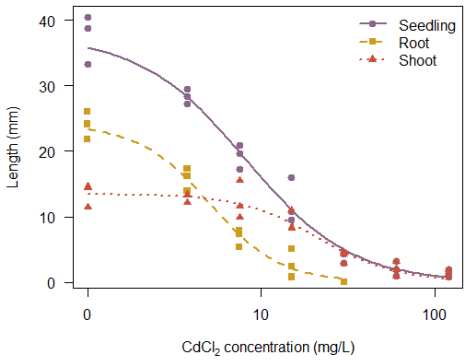
It seems that Cd is most toxic whereas phenol and SDS are least toxic. Both phenol and SDS were closer with regard to their toxicity, especially at lengthy exposure periods. Additional data are available in Appendices (A1 to A3).

2.3.2 Mixture Toxicity Test

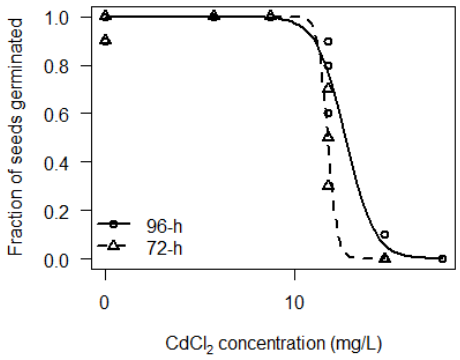
The results revealed less than additive effect of cadmium and phenol on root growth. The mixture IC_{50} (Table 2.15, 2.16) yielded the sum of toxic units (S) greater than 1 (Table 2.14; Fig. 2.7) suggesting a less than



(a) Root, shoot and seedling lengths at 72-hr

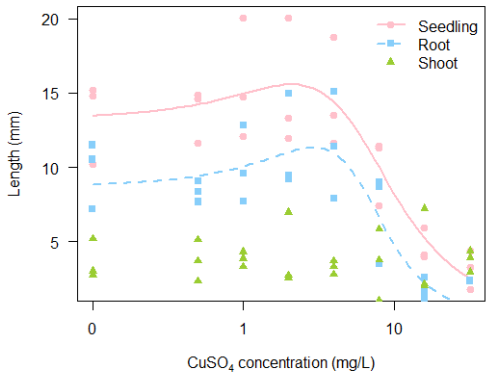


(b) Root, shoot and seedling lengths at 96-hr

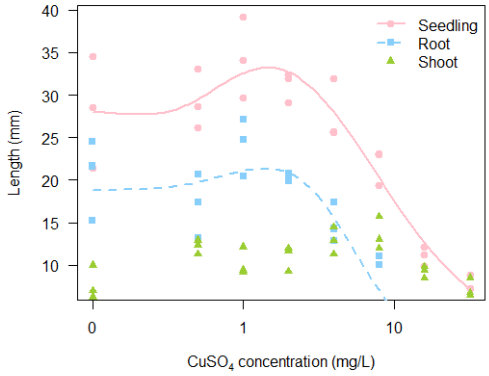


(c) Seed germination at 72- and 96-hrs

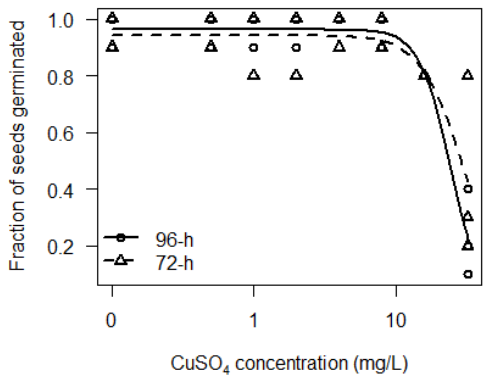
Figure 2.2: Dose-response curves for different morphological end-points of *O. sativa* in $CdCl_2$



(a) Root, shoot and seedling lengths at 72-hr

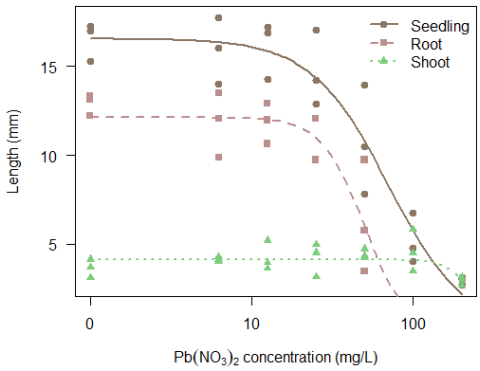


(b) Root, shoot and seedling lengths at 96-hr

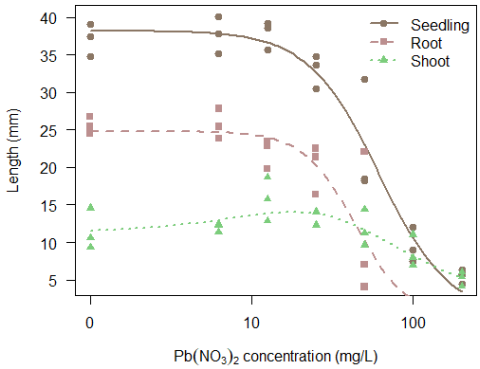


(c) Seed germination at 72- and 96-hrs

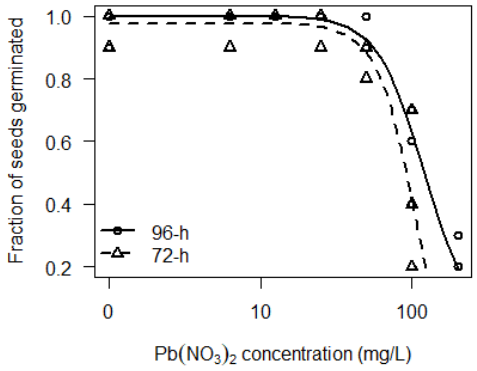
Figure 2.3: Dose-response curves for different morphological endpoints of *O. sativa* in $CuSO_4$. Points without lines denote no significant dose-response relationship



(a) Root, shoot and seedling lengths at 72-hr

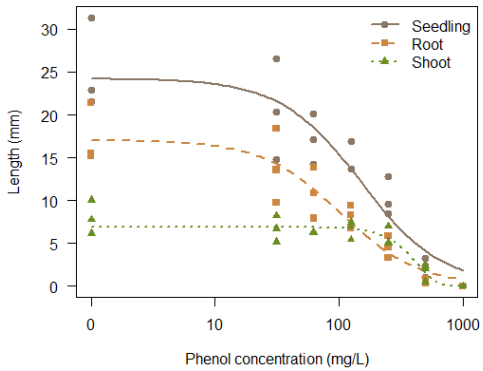


(b) Root, shoot and seedling lengths at 96-hr

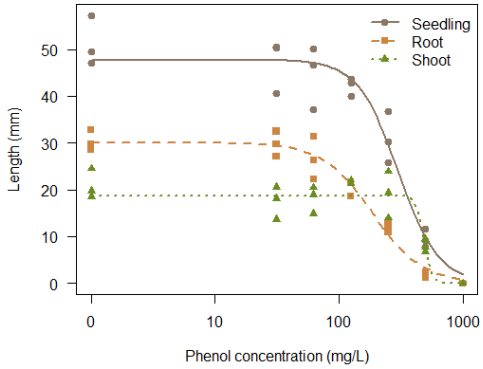


(c) Seed germination at 72- and 96-hrs

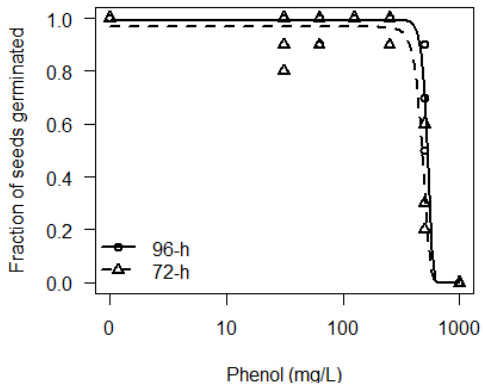
Figure 2.4: Dose-response curves for different morphological endpoints of *O. sativa* in $PbN(O_3)_2$.



(a) Root, shoot and seedling lengths at 72-hr

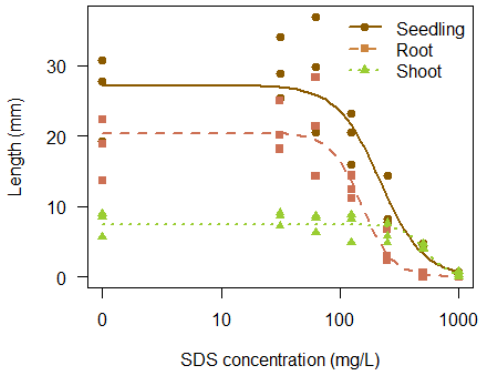


(b) Root, shoot and seedling lengths at 96-hr

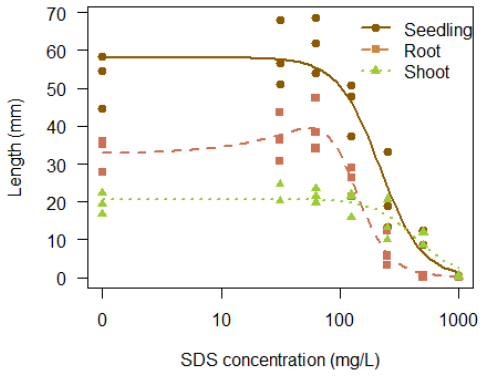


(c) Seed germination at 72- and 96-hrs

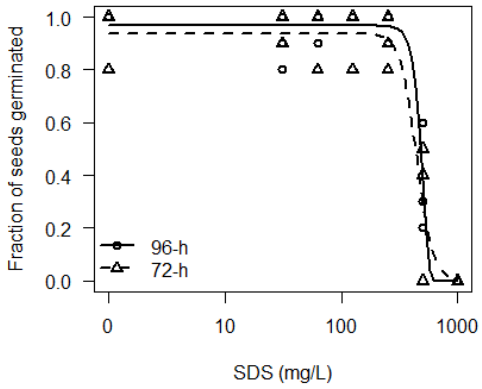
Figure 2.5: Dose-response curves for different morphological endpoints of *O. sativa* in phenol.



(a) Root, shoot and seedling lengths at 72-hr



(b) Root, shoot and seedling lengths at 96-hr



(c) Seed germination at 72- and 96-hrs

Figure 2.6: Dose-response curves for different morphological end-points of *O. sativa* in SDS.

Table 2.9: IC_{50} (LC_{50} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to SDS. BOU = bounds of uncertainty (Delignette-Muller et al., 2011).

Duration	Endpoint	LC/IC_{50}	95% CI		BOU (50%)	
			Lower	Upper	Lower	Upper
96-h	Root	165.6	139	192	62.5	250
	Shoot	434.8	305	565	125	1000
	Seedling	217.4	167	267	125	500
	Germination	481.2	407	556	250	1000
72-h	Root	152.4	113	192	62.5	500
	Shoot	524.5	437	612	125	1000
	Seedling	218.2	148	289	62.5	1000
	Germination	441.4	360	523	250	1000

additive action (antagonism; MTI = -0.42) of compounds present in the mixture. This can also be confirmed by the calculated and Additivity Indices (AI) as their ranges did not overlap zero. The mixture TU (S) for root at 96-h was 2.68 (AI = -1.68), which means that a mixture containing 249.5 mg/L phenol (1.34 TU) and 6.76 mg/L cadmium (1.34 TU) would produce a 50% growth reduction. The individual exposures required lower quantities (5.05 mg/L and 186.2 mg/L, respectively, for cadmium and phenol) than those obtained in the mixture to produce the same effect. Additional data are available in Appendix A

Table 2.10: IC_{25} ($LC_{25}$for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to SDS. BOU = bounds of uncertainty (Delignette-Muller et al., 2011).

Duration	Endpoint	LC/IC_{25}	95% CI		BOU (25%)	
			Lower	Upper	Lower	Upper
96-h	Root	130.4	109	152	62.5	500
	Shoot	273.6	148	399	125	1000
	Seedling	138.9	96.5	181	62.5	1000
	Germination	435.1	206	664	250	500
72-h	Root	109.7	76	143	125	1000
	Shoot	409.9	304	516	125	500
	Seedling	137.8	78.1	198	15	30
	Germination	372.2	250	495	0	500

Table 2.11: NOEC (no observed effect concentration) for different morphological endpoints of *O. sativa* after different durations of exposure to organic and inorganic toxicants.

Toxicant	NOEC (mg/L)							
	Root		Shoot		Seedling		Germination	
	96-h	72-h	96-h	72-h	96-h	72-h	96-h	72-h
$CdCl_2$	< 3.75	3.75	7.5	15	< 3.75	3.75	7.5	7.5
$CuSO_4$	4	8	> 32	> 32	8	8	16	16
$PbN(O_3)_2$	25	25	100	> 200	25	25	50	50
Phenol	62.5	31.3	250	250	125	31.25	250	250
SDS	125	125	250	250	125	125	250	250

2.4 Discussion

The present study focussed on generating toxicity data for some selected toxicants. In general, all the compounds studied elicited toxic responses in a dose-dependent manner. However, the pattern and the extend of toxicity varied with toxicants, exposure duration, and the type of morphological variable (endpoint) considered. A comparison of phytotoxicity of different compounds reported in previous studies is given in Table 2.17. The results show that the sensitivity of *O. sativa* to Cd is several folds lower than those reported for *Spirodela polyrhiza* (Oláh et al., 2014), *Triticum aestivum* (Munzuroglu and Geckil, 2002), and *Cucumis sativus* (Munzuroglu and Geckil, 2002) at 72-h and *Lemna minor* at 96-h (Khellaf and Zerdaoui,

Table 2.12: Ranking (IC_{50} or LC_{50} based) of toxicants in the decreasing order of toxicity to *O. sativa* for two exposure durations.

Endpoint	Sensitivity (IC_{50} or LC_{50} Based)[a]	
	96-h	**72-h**
Root	Cd ⩾Cu>Pb>SDS⩾phenol	Cd>Cu>Pb>phenol>SDS
Shoot*	Cd>Pb>SDS⩾phenol	Cd>Pb>phenol>SDS
Seedling	Cd>Cu>Pb>SDS>phenol	Cu⩾Cd>Pb>phenol⩾SDS
Germination	Cd>Cu>Pb>SDS⩾phenol	Cd>Cu>Pb>SDS⩾phenol

* Cu was not included as the IC_{25} for shoot was greater than the highest concentration (32 mg/L) tested.

[a] = Comparisons using comped function in drc package.

Table 2.13: Ranking (IC_{25} or LC_{25} based) of toxicants in the decreasing order of toxicity to *O. sativa* for two exposure durations.

Endpoint	Sensitivity (IC_{25} or LC_{25} Based)[a]	
	96-h	**72-h**
Root	Cd⩾Cu>Pb>phenol⩾SDS	Cd>Cu>Pb⩾phenol>SDS
Shoot[*]	Cd>Pb>SDS>phenol	Cd>Pb>phenol>SDS
Seedling	Cd>Cu>Pb>SDS⩾phenol	Cd⩾Cu>Pb>phenol⩾SDS
Germination	Cd⩾Cu>Pb>SDS⩾phenol	Cd⩾Cu>Pb>SDS⩾phenol

[*] Cu was not included as the IC_{25} for shoot was greater than the highest concentration (32 mg/L) tested.

[a] = Comparisons using comped function in drc package.

2010). The Cd IC_{50} (for root at 72-h) obtained in the present study is somewhat closer to those obtained for *S. alba root* elongation (Fargašová, 2004). Similarly, the Cu IC_{50} (for root at 72-h) obtained for *O. sativa* is higher than those reported for *S. alba* (Fargašová, 2004) and *Allium cepa* (Arambašić et al., 1995) in previous studies.

Table 2.14: Mixture toxicity index (MTI), sum of toxicity (S), and additivity index (AI) for root length of *O. sativa* after 96-h of exposure.

Variable	Duration	MTI	*S*	AI	AI ranges	
					Lower	Upper
Root	96-h	-0.42	2.68	-1.68	-3.51	-0.51

According to Wang (1994), Cu was found to be more toxic than Cd to *O. sativa* when exposed under darkness for 6 days. The present study, however, indicates that there is no significant difference in toxicity between Cd and Cu when the exposure duration is extended beyond 72 hours under the light. This difference might be attributed to different exposure conditions (light vs dark) or to difference in supporting materials used as the substratum for seeds. It should be noted that in the present study, the rice seeds remained fully submersed in the test solution as no supporting materials were used. However, in Wang's study mentioned above, the rice seeds remained partially submersed because the seeds were germinated on a plastic seed tray. Moreover, heavy metal uptake is influenced by the types of rice cultivars used (Fasahat, 2014), which may also explain the

discrepancies in heavy metal toxicity. Another reason for the difference is the type of metal salts used in the studies (i.e., $CdCl_2$ and $Cucl_2$ in Wang's study vs $CdCl_2$ and $CuSO_4$ in the current study).

Table 2.15: Calculated IC_{50} values (mg/L) for Cd and phenol in mixture.

Compound	Parameter	Duration	IC_{50} (mg/L)	SE	95% CI	
					Lower	Upper
$Phenol_{mix}$	Root	96-h	249.5	2.08	162	355
Cd_{mix}	Root	96-h	6.76	0.05	4.5	9.5

Table 2.16: IC_{50} values (% mixture) obtained for root length of *O. sativa*.

Endpoint	Estimate (%)	SE	95% (CI)	
			Lower	Upper
IC_{10}	11.14	2.7	5.5	16.8
IC_{25}	17.79	3.01	11.5	24.1
IC_{50}	26.8	2.83	20.8	32.7

It is not surprising that Cu produced hormetic effect on shoot at lower concentrations as it is an essential element. Although the hormetic effect on Cu on root was non-significant, the root shows a dose-response curve which indicates a strong tendency towards biostimulation. Wang

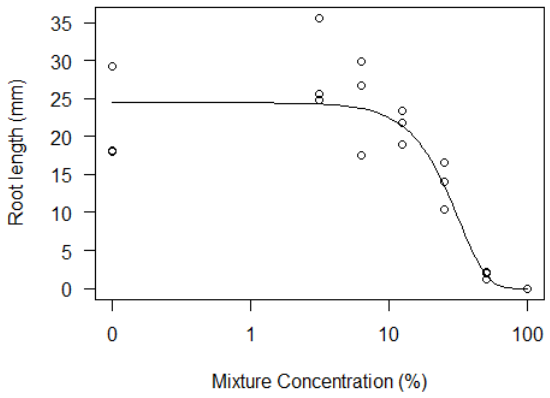


Figure 2.7: Dose-response curve of root exposed to phenol-Cd mixture for 96-h.

could not detect the tendency of Cu to produce hormetic effect at lower concentrations. One reason for this is that the concentration ranges chosen for the study by Wang might not have been appropriate to capture the hormetic effect. It has already been reported that the concentration ranges chosen and the spacing between them (especially at lower ranges) affects the determination of hormesis in bioassays (Belz and Piepho, 2012; Calabrese and Blain, 2011). Cedergreen et al. (2007) observe that hormesis is common in terrestrial plants and that the finding of hormetic response in plants mainly depends on the type of endpoint chosen.

In the case of Pb, none of the morphological responses showed significant differences in IC_{50}s between 72-h and 96-h tests, which indicates that the plant's response to this toxicant is relatively slow compared with other metals studied. This may suggest that *O. sativa* is able to exclude the Pb

to some extent. In fact, rice is reported to be a non-accumulator species (Ashraf et al., 2015). It is hypothesized that callose (which binds with metals) present in the cell wall is one of the main factors that prevents the entry of metals like Pb into the roots (Fahr et al., 2013). Li et al. (2005) have demonstrated that *Arabidopsis thaliana*, when exposed to Pb without seed coat was more sensitive to Pb than those exposed with the seed coat. This suggests that seed coat is a major barrier that prevents the selective uptake of heavy metals by plants. According to Yang et al. (2000), oxalate secreted by the roots also contributes to the tolerance to Pb in rice. Li et al. (2005) suggested that the earlier stages of seed germination are more severely impaired by Cd than Pb, which is in line with the results obtained for *O. sativa* in the present study (as evident from the difference in the IC (or LC) values for different test durations).

With regard to IC and LC values, *O. sativa* was found to be more sensitive to Pb than to phenol. These results contrasts with those obtained for *Lepidium sativum* (Table 2.12) in a previous study (48-hr exposure) in which phenol was recorded more toxic than Pb (Arambašić et al., 1995). However, the pattern of relative sensitivity of *O. sativa* to phenol and Pb was in agreement with those observed in *Allium cepa* (Arambašić et al., 1995).

The SDS IC_{50} (or LC_{50}) values for *O. sativa* was several folds higher than those reported for a unicellular freshwater alga, *Raphidocelis subcapitata* (Liwarska-Bizukojc et al., 2005). Unlike in the case of metals (in which lower concentrations were needed to produce the same level of toxicity as the exposure period extended), organic toxicants (phenol and

SDS) at 96-h required higher concentrations than those required at 72-h to produce the same level of toxicity to *O. sativa*. This unique feature observed for these organic compounds can be useful in toxicity identification evaluation (TIE), in which biological responses unique to toxicants is used to specifically identify toxicants. A decrease in sensitivity of rice to phenol might probably be due to the volatile loss of this compound from the test vessel over time or be due to its uptake and metabolization by the plant.

Seed germination was generally found to be the less sensitive endpoint than shoot elongation (An et al., 2002; Hillis et al., 2011; Hou et al., 2014; Kang and Kong, 2016; Zhi et al., 2015). Contrastingly, seed germination in this study was found to be more sensitive endpoint than shoot elongation for several cases. Rice under, submersed condition, produces plumule earlier than radicle, delaying true germination (He and Yang, 2013). Radicle emergence may, further, be delayed in the presence of toxicants. Since seed germination is operationally defined on the basis of radicle protrusion (1 mm), this delay in radicle protrusion might have resulted in greater sensitivity of seed germination compared with shoot elongation.

A notable aspect of studies shown in the Table 2.12 is that most of them involved exposure of seeds/plants in nutrient medium which is likely to interfere with toxicant bioavailability. This can be one of the major sources of disparities between different studies. It is an established fact that the nutrients in the culture solution can modify the metal toxicity (Fjällborg et al., 2006; Kopittke et al., 2010; Rout and Das, 2003). *O. sativa* can be used successfully in toxicity tests as it does not require

nutrient solution for germination. Besides this, the length of exposure can also influence the toxicity.

The study also compared low effect concentrations (IC_{25}), NOECs, and BOUs to assess their adequacy in ecotoxicity studies. In several cases, NOECs fell below the calculated 95% confidence intervals of IC_{25}; but none of them fell above the upper limits. This indicates that IC_{25} may underestimate the toxicity at low effect levels at least in some cases. It could be inferred from the results that NOECs still remains protective when compared with IC_{25} as the safest concentration. It is also evident that the BOUs obtained from simultaneous confidence intervals bridges the gap between IC_x values and NOECs. Although BOUs were much wider in several cases, it provided sufficient information on the extend of toxicity in the case where NOECs and IC_{25} did not match well. The problems associated with depending solely on one estimate can thus be overcome by including multiple estimates such as NOECs, IC_xs, and BOU in ecotoxicity studies.

In a previous study (SU et al., 2008), the joint toxicity of substituted phenol and cadmium to *Phtobacterium phosphoreurm* – a gram negative bacteria, has shown to be simple addition. Their study using combinations of Cd and phenol at various dilutions (Cd: 0.2, 0.5 and 0.8 EC_{50}; phenol: 3.11, 3.42 and 3.77 -logEC_{50}) yielded additivity indices ranging from 0.05 to 0.14. It was also revealed that there was a strong correlation between the joint toxicity of substituted phenols under different concentrations of Cd and identical descriptors, the heat of formation (ΔHf) and log *n*-octanol/water partition coefficient (lgP). The present study reveals that

the action of Cd and phenol mixture on *O. sativa* is less than additive, which is in contrary to those observed in *Phtobacterium phosphoreurm* as described above. The reason for this might be the fact that the sensitivity to the same toxicant varies across the species (Eaton et al., 2006) and that the prokaryotes and eukaryotes differ in their genetic mechanism of toxic response (Gutiérrez et al., 2015). Such variations create issues when different species are used in the prediction of risk posed by chemical mixtures (Gregorio et al., 2013). It was also demonstrated that phenol and metals like copper could form a non-toxic complex and follow an independent mode of action (Kim et al., 2006). In fact, phenol is formed in plants during the metabolism. Generally, the metal ions could complex with organic compounds leading to reduced toxicity (Jin et al., 2014). Phenol is less toxic to plants at lower concentrations because of metabolic advantage that plants possess to degrade phenol (Ucisik and Trapp, 2006). The hydroxyl and carboxyl groups of phenolic compounds can bind to metal ions and chelate them (Jun et al., 2003). Organometallic complexes formed from some phenolic compounds secreted by plants are also known to raise the bioavailability of micro and macro nutrients (Becker et al., 1998), which may also explain the reduction in toxicity.

The assessment of the combined effect of phenol and Cd revealed that in combination these toxicants act antagonistically. Though the formation of organometallic complex can be attributed to the reduced toxicity of phenol-Cd mixture, the reason behind the hormetic effect remains unclear and needs further investigation. The study also stresses the importance of bioassay of compounds in combination than individually.

Several studies suggest that plants should be included in tired assessments. *O. sativa* is one of the few plants which germinate anaerobically by coleoptile elongation; this makes them better adapted to soil-less germination compared with other recommended test species like lettuce which is widely used in bioassays. Furthermore, *O. sativa* belongs monocotyledons, a class of angiosperm, least represented in ecotoxicological bioassays. Rice seeds have been shown to remain viable up to 3 years under storage (Wang and Keturi, 1990). It has already been proven that the supporting materials used in bioassays alter the sensitivity of the test species to the toxicants. Though several methods have been devised to overcome this problem, they are not as simple and cost effective as the hydroponic method which does not require any supporting material. Another problem associated with root elongation bioassays is the considerable amount of time consumed in the manual measurement of root length which also poses the risk of human exposure to toxicants. The use of image analysis software in the measurement of morphometric endpoints can overcome this issue. The anaerobic method of rice seed germination can be considered a better choice in phytotoxicity tests due to its simplicity, cost effectiveness and versatility.

2.5 Conclusion

The present study points out that, despite the differences observed in sensitivity between *O. sativa* and other standard test species, the simplicity in toxicity tests with *O. sativa* makes it a valuable tool in the toxicity assessment of environmental samples. Further, the study highlights the

importance of including multiple endpoints and/or multiple time points in toxicity tests. The study has also generated phytotoxicity data of SDS for which limited data exist for vascular plants. Cadmium was found to be the most toxic among the inorganic toxicants, whereas SDS and phenol were found to more or less similar, but least toxic among the organic toxicants compared with inorganic toxicants. The phytotoxicity data generated for the reference toxicants can be useful in routine toxicity test. As opposed to the general findings in other studies that germination is the least sensitive endpoint, this study demonstrated that the sensitivity of different endpoints including germination varies with regard to the nature of toxicant and also with the duration of exposure. This differential response of endpoints, unique to particular classes of toxicants, can be useful in the characterisation of toxicants to specific classes. The study also concludes that the wealth of information in toxicity tests can be improved by including multiple endpoints, multiple estimates of toxicity, and multiple exposure durations. Bioassays using Cd-phenol mixture showed that these compounds act antagonistically to root. The results signify the importance of toxicity tests using chemical mixtures rather than relying solely on toxicity tests using individual toxicants.

Table 2.17: Endpoint estimates obtained in previous studies for some selected plant species exposed to organic and inroganic compounds.

Species	Compound	Medium	Duration	Endpoint	Response	Concentration	Reference*
Sinapis alba	$CdCl_2$	NS, FP	3 d, NL	EC_{50}	RE	48 mg/L (34.56 - 51.83)[a]	1
Sinapis alba	$CdCl_2$	NS, FP	3 d, NL	LC_{50}	SG	692 mg/L (630.9 - 717.2)[a]	1
O. sativa	$CdCl_2$	DiH_2O, ST	6 d, D	IC_{50}	RW	1.4 mg/L (1.3 -1.5)[a]	2
Spirodela polyrhiza	$3\,CdSO_4 \cdot 8\,H_2O$	NS	3 d, AL	IC_{50}	GI	0.297 mg/L (0.209 - 0.385)[a]	3
Spirodela polyrhiza	$3\,CdSO_4 \cdot 8\,H_2O$	NS	5 d, AL	IC_{50}	GI	0.080 mg/L (0.064 - 0.096)[a]	3
Lemna minor	$CdCl_2$	NS	4 d, AL	EC_{50}	GI	0.91 mg/L	4

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Table 2.17 – *Continued from previous page*

Species	**Compound**	**Medium**	**Duration**	**Endpoint**	**Response**	**Concentration**	**Reference**
Triticum aestivum	$CdCl_2 \cdot H_2O$	ddH_2O, FP	1d, D	EC_{50}	SG	1.5 mM	5
Cucumis sativus	$CdCl_2 \cdot H_2O$	ddH_2O, FP	1d, D	EC_{50}	SG	4.0 mM	5
Triticum aestivum	$CdCl_2 \cdot H_2O$	ddH_2O, FP	3 d, D	EC_{50}	SG	2.0 mM	5
Cucumis sativus	$CdCl_2 \cdot H_2O$	ddH_2O, FP	3 d, D	EC_{50}	SG	$>$ 8.0 mM	5
Lemna minor	$CdCl_2$	NS	7 d, AL		FN	0.323 mg/L (0.232 - 0.450)[a]	6
Triticum aestivum	$CdCl_2 \cdot 2.5\,H_2O$	NS	6 d AL	EC_{50}	RE	4.32 µM	7
Lactuca sativa	Cd, Metallic	ddH_2O	2 d, AL	EC_{50}	RE	0.132 mg/L (0.059 - 0.181)[a]	8
Sinapsis alba	$CdCl_2 \cdot 2.5\,H_2O$	NS, FP	3 d, NL	IC_{50}	RE	5.85 mg/L (4.28 - 6.12)[a]	9

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Table 2.17 – *Continued from previous page*

Species	**Compound**	**Medium**	**Duration**	**Endpoint**	**Response**	**Concentration**	**Reference**
Lemna gibba	$CuSO_4 \cdot 5\,H_2O$	NS	4 d, AL	EC_{50}	FA	0.45 mg/L	10
Lactuca sativa	$CuSO_4$	DiH_2O	4 d, D	EC_{50}	RE	3000 µg/L	11
Lemna gibba	$CuSO_4$	NS	2 d, AL	EC_{50}	RE	310.0 µg/L (236.7 - 391.3)[a]	12
Lactuca sativa	Cu, Metallic	ddH_2O	2 d, AL	EC_{50}	RE	0.109 mg/L (0.102 - 0.17)[a]	8
Oryza sativa	$Cucl_2$	DiH_2O, ST	6 d, D	IC_{50}	RE	0.22 mg/L (0.2 - 0.25)[a]	2
Allium cepa	$CuSO_4$	TW	2 d, NL	IC_{50}	RE	0.00112 mM/L ± 0.00019[b]	13
Lepidium sativum	$CuSO_4$	TW, FP	2 d, NL	IC_{50}	RE	2.42917 mM/L ± 0.25897[b]	13

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Table 2.17 – *Continued from previous page*

Species	**Compound**	**Medium**	**Duration**	**Endpoint**	**Response**	**Concentration**	**Reference**
Sinapsis alba	$CuSO_4 \cdot 5\,H_2O$	NS, FP	3 d, NL	IC_{50}	RE	2.02 mg/L (1.71- 2.48)[a]	9
Sinapsis alba	$Pb(NO_3)_2$	NS, FP	3 d, NL	IC_{50}	RE	101.32 mg/L (96.7 -106.3)[a]	9
Allium cepa	$Pb(NO_3)_2$	TW	2 d, NL	IC_{50}	RE	0.03976 mM/L ± 0.00380[b]	13
Lepidium sativum	$Pb(NO_3)_2$	TW, FP	2 d, NL	IC_{50}	RE	3.37130 mM/L ± 0.87418[b]	13
Allium cepa	Phenol	TW	2 d, NL	IC_{50}	RE	3.02166 mM/L ± 0.04811[b]	13
Lepidium sativum	Phenol	TW, FP	2 d, NL	IC_{50}	RE	0.86264 mM/L ± 0.02498[b]	13
Lactuca sativa	Phenol	ddH_2O	2 d, AL	EC_{50}	RE	0.124 mg/L (0.062 - 0.148)[a]	8

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Table 2.17 – *Continued from previous page*

Species	**Compound**	**Medium**	**Duration**	**Endpoint**	**Response**	**Concentration**	**Reference**
Panicum spp.	Phenol	Solution, FP	5 d, D	EC_{50}	RE	120 mg/L	14
Allium cepa	Phenol	TW	4 d, AL	EC_{50}	RE	9×10^{-5} M	15
Raphidocelis subcapitata	SDS	NS	3 d, AL	IC_{50}	CD	36.58 mg/L	16

[a] 95% confidence interval.

[b] Standard error.

NL = Natural light; AL = Artificial light; D = Darkness; FP = Filter paper; ST = Seed tray; ddH_2O = double distlled water; ddH_2O = Deionised water; TW = Tap water; RE = Root elongation; RW = Root Weight; SG = Seed germination; FA = Frond area; CD = Cell density; FN = Frond number; GI = Growth inhibition.

[*] 1 (Fargašová, 1994) 2 (Wang, 1994) 3 (Oláh et al., 2014) 4 (Khellaf and Zerdaoui, 2009) 5 (Munzuroglu and Geckil, 2002) 6 (Naumann et al., 2007) 7 (Cao et al., 2007) 8 (Park, Yoon, et al., 2016) 9 (Fargašová, 2004) 10 (Khellaf and Zerdaoui, 2010) 11 (Fjällborg et al., 2006) 12 (Park, Kim, et al., 2013) 13 (Arambašić et al., 1995) 14 (Wang, 1986) 15 (Siddiqui et al., 2011) 16 (Liwarska-Bizukojc et al., 2005).

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Chapter 3

Toxicity Identification Evaluation (TIE) of a Chemical Mixture with *Oryza sativa*

3.1 Introduction

It was the clean water Act (CWA) of 1972 and subsequent amendments which lead to the implementation of approaches to control the release of pollutants into the water bodies of USA. The earlier approach of pollutant control was purely "technology-based", which simply relied on the extent to which the existing technology could remove pollutants. Also, it did

not cover the majority of pollutants and thus did not guarantee sufficient protection of surface waters.

Later, as per the US Federal Register (49:9016, 1984), a new policy was issued which resulted in the incorporation biological methods called whole effluent toxicity (WET) tests in monitoring process (Ankley et al., 2011). However, the WET method measures toxicity rather than identifying the cause of toxicity. A mere knowledge about the effluent being toxic or not does not provide any information about the pollutants responsible for the toxicity so that proper treatment methods could be implemented accordingly (Ankley et al., 2011). This necessitated the development of a novel method to specifically identify the toxicants present in the aqueous sample, which is later known as toxicity identification evaluation (TIE).

Before TIE was introduced, the conventional approach (Fig.3.1) of identifying toxicants relied mainly on "priority pollutants" which covered only a few among the wide variety of compounds (USEPA, 1991). In this approach, both chemical as well as toxicity data are compared with the literature data to identify toxicants. Most of the conventional methods of identifying toxicants, however, failed mainly because the improper methods of sample handling and manipulations which resulted in either loss of toxicity or artefactual toxicity (Ankley et al., 2011). The conventional water quality parameters may not always detect real culprit of toxicity because the toxicant concentration in the effluent may be at concentrations much lower than the detection limit. Besides this, the effluent toxicity usually tends to show wide temporal variation. Additionally, there is no guarantee that the same toxicant is causing toxicity in a particular

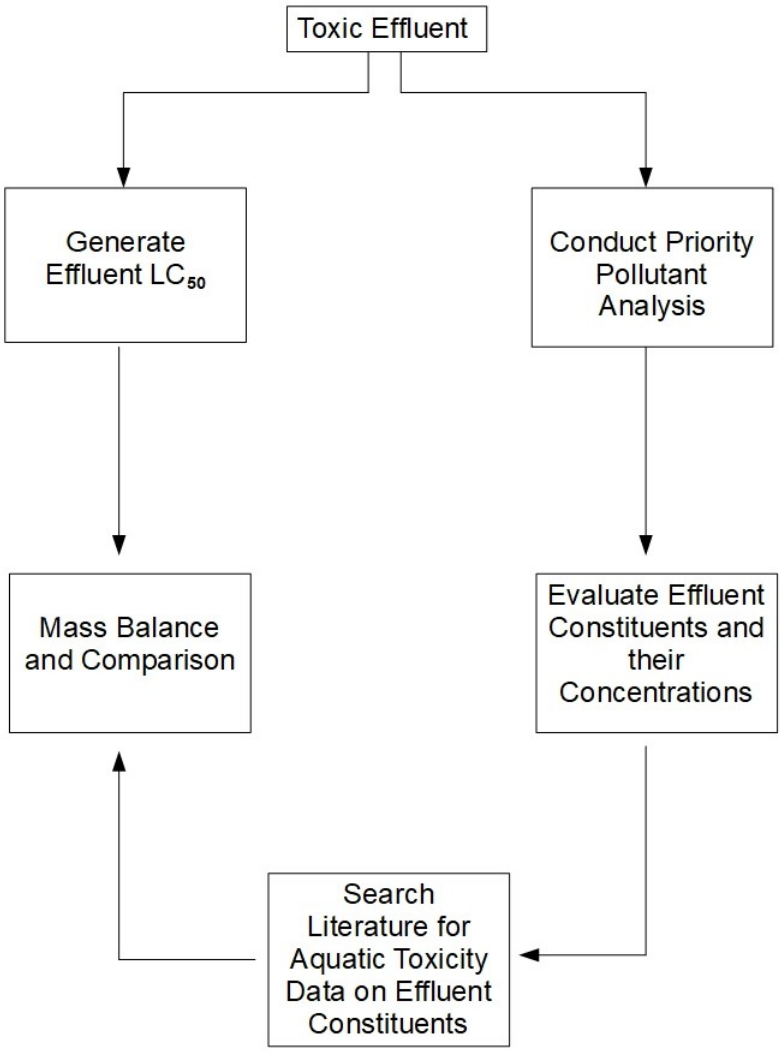


Figure 3.1: The conventional approach to TIE (USEPA, 1991)

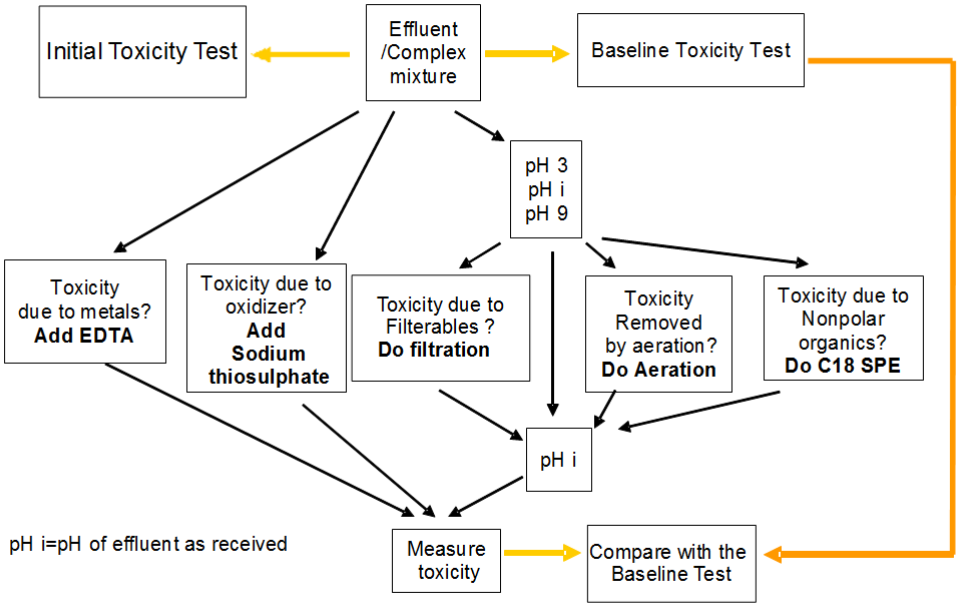


Figure 3.2: Work flow of Effluent Toxicity Identification Evaluation (USEPA, 1991)

effluent over time. Above all, the conventional way of TIE fails when the toxicant is a non-priority pollutant as it is practically impossible and costly to screen the sample for every compound in search of a toxicant (USEPA, 1991).

The recent, toxicity based approach (Fig. 3.2) to TIE uses the biological response elicited by TIE manipulations to detect the presence of toxicants. The modern approach to TIE involves three phases: toxicity characterisation (Phase I), toxicity identification (Phase II), and toxicity confirmation (Phase III). In phase I, the effluent (or receiving water) is bioassayed before and after certain physical and chemical manipulations, and the results are compared. The manipulations involve filtration, aeration, and C_{18} SPE (solid phase extraction) of pH adjusted/maintained (low, high, and initial pH) aliquots of samples with subsequent back adjustment to the initial pH value. Some other aliquots undergo pH graduation (pH 7, 8, and 9), EDTA addition, and sodium thiosulfate (STS) addition. All these aliquots are then, bioassayed. Based on the results of Phase I bioassays, the toxicants can be characterized into filterables, volatiles, non-polars, pH dependents, chelatables, or oxidants etc.

The results from phase I manipulations (Fig. 3.2) determines the type of manipulations to be performed in Phase II. For example, the lack of toxicity in the post column sample from SPE in relation to baseline suggests the presence of non-polar organics or cationic metals. This necessitates more intensive fractionation of samples through HPLC, followed by bioassays or metal analysis. Since the solvents used in Phase II fractionation (e.g., methanol, acetonitrile, acetone, isopropanol) is relatively

less toxic, toxicity tests can be directly performed on the SPE/HPLC eluates (USEPA, 1993a). Phase III uses a "weight of evidence" based approach (USEPA, 1993b) to detect the toxicant and involves the evaluation of the following:- correlation of temporal change in toxicity with the concentration of suspect toxicant, relative sensitivity of different species to the sample toxicity, specific symptoms displayed by the organisms, response of the organisms to the addition or deletion of suspect toxicant to the sample, toxicity of toxic fractions added back to the post SPE column.

Like any other technology, TIE too has some limitations. One of the main limitations of TIE is that it identifies only the toxicants. TIE fails to identify other stressors such as invasive species, eutrophication, shifts in hydrological regime etc. Additionally, no known techniques exist in TIE to identify PPCP (pharmaceuticals and personal care products), and the intermediates of pesticide and herbicide degradation (Ho and Burgess, 2013). Recent researches are advancing to tackle all these issues.

A wealth of data on TIEs using animals such as *Daphnia*, fishes, sea urchins, and clams exists in the literature. However, there is a scarcity of data on TIEs using plants. Although a few TIE studies using algae such as *Selenastrum capricornutum*, *Nitzschia closterium*, *Isochrysis galbana*, and *Ulva pertusa* are available, a majority of them represent marine studies (Deanovic et al., 1999; Hogan et al., 2005; Kim, Han, et al., 2015; Strom et al., 2009). Furthermore, an insufficient amount of data exists with regard to TIEs using vascular plants. The only available reports come from studies using *Lactuca sativa* (Fjällborg, Li, et al., 2006) and

Raphanus sativus (Villamar et al., 2014).

The aim of the present study is to develop and validate the Toxicity Identification Evaluation (TIE) protocol for the liquid sample with a vascular plant *Oryza sativa*.

3.2 Materials and Methods

The experiments were conducted in two steps. At first, the TIE manipulations have to be standardized for the test species *O. sativa*. The second step involved the validation of TIE using a synthetic (binary) mixture. The first step in TIE was, therefore, to determine the range of tolerance of test species to TIE manipulations (standardised). For this, bioassays were performed with *O. sativa* var. Jyothi in distilled water which received physical or chemical manipulations of TIE. The bioassays were performed in triplicate at 28±2°C with a 14:10 hour light:dark cycle (300 µmol/m^2/s) for a duration of 72 and 96 hours. Each petri dish contained 10 ml of sample and 10 rice seeds. All TIE manipulations were based on guidelines given by USEPA (1991). Different Phase I manipulations are given below.

3.2.1 Tolerance of *Oryza sativa* to Chemical Manipulations in Phase I TIE

Toxicity tests were conducted to determine the concentration ranges of Phase I chemicals that the test species tolerates. This involved the exposure of *O. sativa* var. Jyothi to different levels of EDTA (0, 12.5, 25, 50, 100, 200, and 400 mg/L), STS, i.e. $Na_2S_2O_3$ (0, 281.25, 562.5, 1125, 2250, 4500, 9000 mg/L), methanol (0, 0.389, 0.779, 1.559, 3.118,

6.237, 12.475 %), and pH (distilled water with pH adjusted to 3 and 11, kept for 24-hr, and returned to initial pH). Graduated pH tests were also performed with pH adjusted (pH 7, 8, and 9) distilled water without back adjustment. Root, shoot, and seedling lengths and seed germination were measured using Fiji software (Schindelin et al., 2012).

3.2.2 Tolerance of *Oryza sativa* to Physical Manipulations in Phase I TIE

In physical manipulations, *O. sativa* was exposed to different aliquots of distilled water which had undergone different physical manipulations (aeration, filtration, and C_{18} SPE) after adjustment of pH to 3 and 11 (or pH 9 in the case of C_{18} SPE) followed by back adjustment to initial pH. A lower pH value was chosen for C_{18} SPE as the higher pH would damage the column.

3.2.3 TIE of Chemical Mixture

For TIE, a chemical mixture of phenol and Cd each at their 5 X IC 50 (25.25 mg/L) for root (at 96-h) was used (the same combination of compounds used for combined effect test mentioned in chapter 2). The chemical mixture also underwent both physical and the chemical manipulations of TIE (after being split into different aliquots). A baseline test (unmanipulated sample) consisting of a mixture with 100, 50, 25, 12.5, 6.25, and 0% concentration was also run concurrently to compare the changes in toxicity caused by TIE manipulations.

Chemical manipulations involved addition (dilution approach) of

EDTA (i.e., 0.5 X IC_{50} for root = 109 mg/L), STS (i.e., 0.5 X IC_{50} for root = 2542 mg/L), and pH adjustment to a value of 3 and 11. In graduated pH test, the pH was elevated to a value of 7, 8, and 9 (without back adjustment). Physical manipulations involved aeration (1 hour), filtration (Whatman glass fibre filter; 0.45 µm), and C_{18} SPE (Waters, 200 mg) of samples adjusted to pH 3 or 11 (or pH 9 in the case of C_{18} SPE).

Except for graduated pH test, all the samples were adjusted back to initial pH (4.6) before they were used for bioassay. For the C_{18} SPE, a portion of the filtered sample was used. All samples were equilibrated for 24 hours before the seeds were introduced. All the TIE manipulations were followed by toxicity tests. The experimental conditions were the same as those used in the standardization step. All the measurements (seed germination and, root, shoot, and seedling lengths) were done with Fiji software.

3.2.4 Data Analysis

The computation of IC_{50} and IC_{25} estimates (LC_{50} and LC_{25} in the case of seed germination) were done using drc package (Ritz et al., 2015). IC values of baseline test were compared to those obtained from TIE manipulations. NOECs were calculated by multiple comparisons (one-tailed) between control and treatment (EDTA, STS, and methanol) concentrations using multcomp package (Hothorn et al., 2008).

3.3 Results

3.3.1 Effect of Chemical Manipulations of TIE on Toxicity to *Oryza sativa*

a) EDTA

For the 96-h exposure, hormetic model (4 parameter Cedergreen-Ritz-Streibig model) was found to be the best fit model for root, whereas Weibull (3-parameter, type 2) model was found to be the best fit for seedling (Fig. 3.3b). Log-logistic (3-parameter) model was found to be the best fit for both root length and seedling length at 72-h (Fig. 3.3a; Appendix B1). Dunnet's multiple comparisons, however, failed to detect any hormetic effect in the case of root (Table 3.3; Appendix B3). EDTA treatment did not cause any significant ($p > 0.05$) effect on shoot growth (Table 3.1, 3.2) as well as on seed germination (IC_{50} and $IC_{25} > 400$ mg/L) even after 4 days of exposure. Root elongation was greatly affected by EDTA treatment (IC_{50} of 156.2 and 151.7 mg/L for 96-h and 72-h, respectively). However, the IC_{50} values for root did not differ significantly across the two exposure durations. The seedling length was the only endpoint which produced a statistically lower IC_{25} than the corresponding IC_{50} both at 96-h and 72-h. The NOECs for both root and seedling were in perfect agreement with the corresponding IC_{25}s, as they fell within the 95% CI of IC_{25} (Table 3.3).

Table 3.1: Tolerance (intrinsic toxicity) of *Oryza sativa* to chemical manipulations of TIE. IC/LC_{50} (in bold) and 95% CI.

Compound	Duration	Endpoints			
		Root	Shoot	Seedling	Germination
EDTA (mg/L)	96-h	**218.1**	**> 400**	**336.6**	**> 400**
		137.6 - 298.6	-	213.7 - 459.5	-
	72-h	**233.3**	**> 400**	**360.2**	**> 400**
		169.2 - 297.4	-	250.3 - 470.0	-
STS (mg/L)	96-h	**5084**	**8752**	**5974**	**> 9000**
		3122 - 7047	4672 - 12833	3783 - 8165	-
	72-h	**5059**	**> 9000**	**6679**	**> 9000**
		3482 - 6636	-	4595 - 8764	-
Methanol (%)	96-h	**1.72**	**1.45**	**1.62**	**3.24**
		0.83 - 2.61	0.90 - 2.01	0.98 - 1.65	0.95 - 2.28
	72-h	**1.25**	**1.39**	**1.32**	**1.66**
		0.89 - 1.61	0.88 - 1.91	0.98 - 1.65	1.31 - 2.02

b) STS

Brain-Cousens hormesis model (4-parameter) was found to be the best fit model for all the endpoints, except for shoot growth at 96-h (Fig. 3.3d; Appendix B1). The 72-h exposure produced a significant ($p < 0.05$) hormetic effect at 1125 mg/L (Dunnet's multiple comparisons) for both root and seedling (Fig. 3.3c; Appendices B1 to B7). However, after 96-h exposure, the hormetic effect ($p < 0.05$) for the seedling shifted to the next higher level of concentration (2250 mg/L).

The seed germination remained unaffected (Table 3.1, 3.2) by STS both at 96-h and 72-h (IC_{50} and IC_{25} > 9000 mg/L). For the other endpoints (root, shoot, and seedling), the 96-h IC_{50}s did not differ significantly among one another (Table 3.1). The STS did not inhibit shoot growth until 72-h. For the shoot growth at 96-h, the IC_{25} and IC_{50} were 3804 mg/L and 8752 mg/L, respectively, whereas, at 72-h, the corresponding IC values were > 9000 mg/L. The IC_{25}s for the shoot length at 96-h and the seedling length at 72-h were significantly lower than the corresponding IC_{50} values; nevertheless, the NOECs (Table 3.3) for these endpoints fell below the 95% CI of IC_{25}.

c) Methanol

Except for shoot length at 72-h, all other dose-response curves were fitted using Weibull (3-parameter, type 2) model (Fig. 3.4a; Appendix B1). Unlike the other manipulations, methanol caused a significant inhibitory effect on seed germination. However, the impact of methanol on seed germination (Fig. 3.5) was generally low when compared with its effect on root, shoot and seedling lengths. At 72-h, none of the point estimates (IC_{50} and IC_{25}) differed significantly among the endpoints studied. However, as the exposure duration increased, a clear distinction was observed in the point estimates of different endpoints. The 96-h IC_{50}s were 1.14, 0.85, 1.07 and 2.41% for root, shoot, seedling, and germination, respectively (Fig. 3.4b; Table 3.1, 3.2).

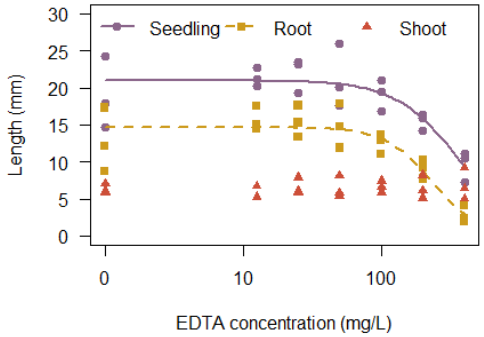
Table 3.2: Tolerance (intrinsic toxicity) of *Oryza sativa* to chemical manipulations of TIE. IC/LC$_{25}$ (in bold) and 95% CI

Compound	Duration	Endpoints			
		Root	**Shoot**	**Seedling**	**Germination**
EDTA	96-h	**156.2**	**> 400**	**172.3**[b]	**> 400**
(mg/L)		95.5 - 216.9	-	107.0 - 237.6	-
	72-h	**151.7**	**> 400**	**202.3**[b]	**> 400**
		88.6 - 214.8	-	118.9 - 285.7	-
STS	96-h	**3646**	**3804**[b]	**3826**	**> 9000**
(mg/L)		2524 - 4767	2401 - 5207	2722 - 4930	-
	72-h	**3586**	**> 9000**	**4108**[b]	**> 9000**
		2662 - 4511	-	3114 - 5102	-
Methanol	96-h	**1.14**	**0.85**	**1.07**	**2.41**
(%)		0.44 - 1.84	0.36 - 1.35	0.67 - 1.28	1.67 - 3.16
	72-h	**0.99**	**0.7**[b]	**0.98**	**0.87**
		0.63 - 1.34	0.22 - 1.19	0.67 - 1.28	0.59 - 1.15

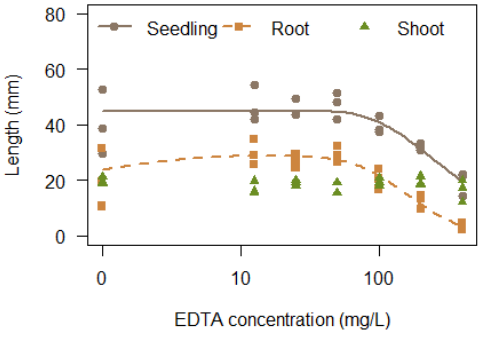
[b] = significantly lower than the corresponding IC/LC$_{50}$ ($p < 0.05$). .

d) pH Adjustment and Graduated pH

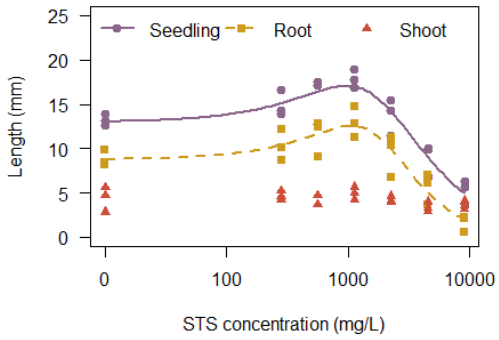
The other chemical manipulations including pH adjustment test (at pH 3, and 11 with back adjustment to initial pH) and graduated pH test (at pH 7, 8 and 9) did not show any significant differences in the endpoints studied for both the test durations (Fig. 3.6). Though there was a slight tendency of increase in growth at higher pH values in the pH adjustment tests, the differences were not significant enough to confound the results.



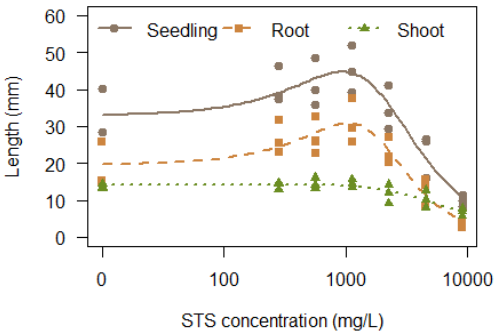
(a) EDTA treatment at 72-h.



(b) EDTA treatment at 96-h.



(c) STS treatment at 72-h.



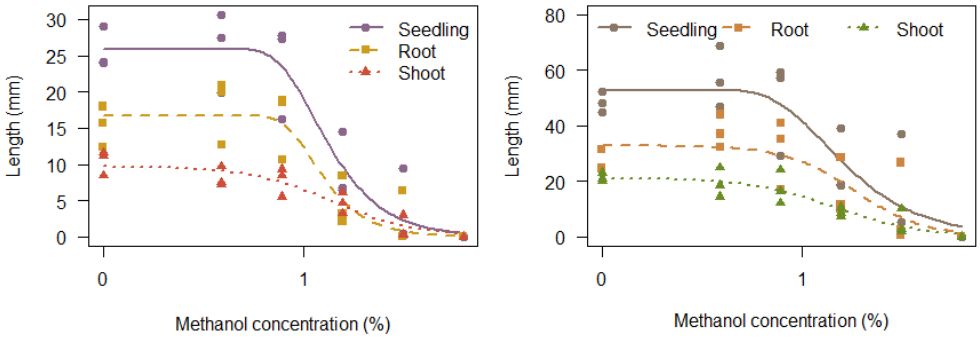
(d) STS treatment at 96-h.

Figure 3.3: Dose-response curves showing the tolerance of *O. sativa* to chemical manipulations in TIE. Points without line represent endpoints with no significant dose-response relationship.

3.3.2 Effect of Physical Manipulations (aeration, filtration, C_{18} SPE) of TIE on Toxicity to *Oryza sativa*

None of the physical manipulations caused a significant difference ($p > 0.05$) in any of the endpoints studied for both the exposure periods. The aeration, filtration and SPE tests produced results similar to those observed in pH adjustment tests (slight, but non-significant growth reduction at lower pH values for all the endpoints).

None of the endpoints was significantly different in graduated pH, although there was a slight reduction in response at both the extremes of pH (results not shown).



(a) Methanol treatment at 72-h. **(b)** Methanol treatment at 96-h.

Figure 3.4: Dose-response curves showing the tolerance of *O. sativa* to chemical manipulations in TIE.

Table 3.3: NOEC values for chemicals used in TIE manipulations.

Compound	Duration	NOEC			
		Root	**Shoot**	**Seedling**	**Germination**
EDTA	96-h	200	> 400	200	> 400
(mg/L)	72-h	200	> 400	200	> 400
STS	96-h	4500	2250[b]	4500	> 9000
(mg/L)	72-h	4500	> 9000	2250[b]	> 9000
Methanol	96-h	3.118[a]	0.779	1.559	1.559[b]
(%)	72-h	0.779	0.779	0.779	0.779

[a] significantly higher than the corresponding IC/LC_{25}.

[b] significantly lower than the corresponding IC/LC_{25}.

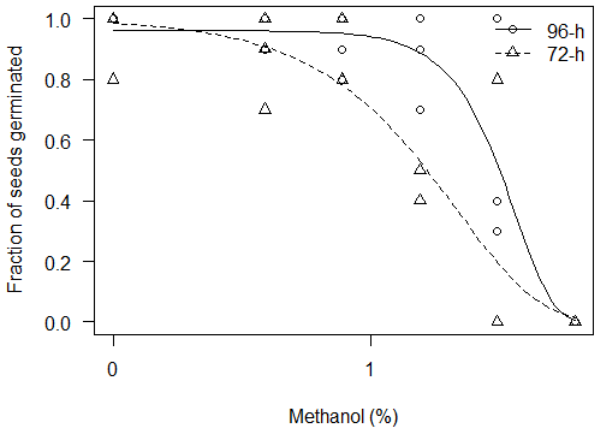


Figure 3.5: Dose-response relationship of seed germination in methanol.

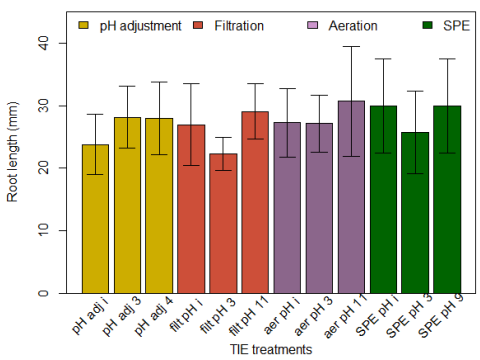
3.3.3 TIE with Chemical Mixture

The IC/LC_{50}s for baseline mixture (at 5 X 96-h IC_{50} of Cd and phenol for root) ranged from 25.8, 49.6, 35.2, and 59.3% for root length (Table 3.4; Fig. 3.7), shoot length (Table 3.5; Fig. 3.8), seedling length (Table 3.6; Fig. 3.9) and seed germination (Table 3.7), respectively at 96-h.

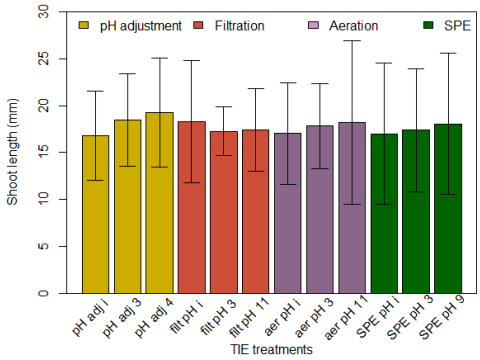
In general, EDTA, aeration at pH 11, C_{18} SPE at pH 9 and graduated pH test at pH 9 significantly reduced the toxicity of chemical mixture to root. None of the TIE manipulations produced any significant detectable change in seed germination. Toxicity for root decreased approximately in the same order of magnitude in EDTA, SPE at pH 9, pH adjustment at pH 3, graduated pH at pH 9, and Aeration at pH 11 treatments.

In the case of root, both EDTA and STS additions resulted in similar IC_{50}s ($\simeq$ 35%; higher than baseline IC_{50}), but the latter was not significant ($p > 0.05$). Similar response pattern was observed for shoot and seedling, in which case the EDTA produced a significant improvement in growth, whereas STS did not. Nevertheless, for all the endpoints, IC values for STS treated samples were considerably closer to those observed for EDTA. TUs (based on root IC_{50}) for all those TIE treatments supposed to lower the toxicity were reduced to $\simeq$ 3 when compared with baseline TU for root (TU $\simeq$ 4).

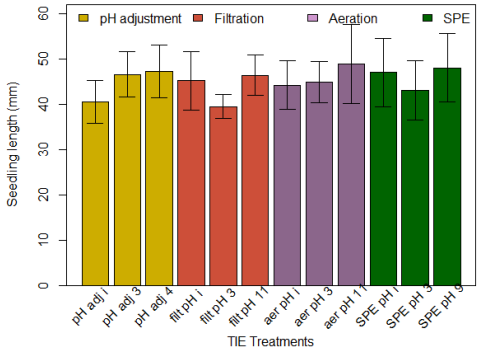
None of the physical manipulations at lower pH (ie, aeration, filtration, and SPE, at pH 3) caused a significant reduction in toxicity for any of the endpoints studied. By contrast, with the exception of filtration, all the physical manipulations (aeration and SPE) at higher pH followed by



(a) Methanol treatment at 72-h.



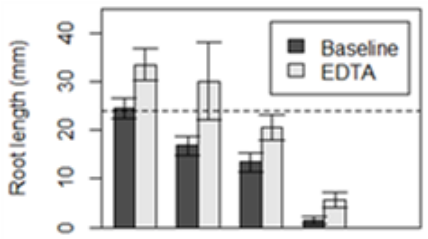
(b) Methanol treatment at 96-h.



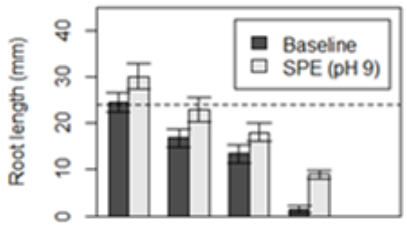
(c) Methanol treatment at 96-h.

Figure 3.6: Effect of TIE manipulations (pH change, aeration, SPE, and filtration) with distilled water on *Oryza sativa*. All samples were adjusted back to initial pH before bioassay.

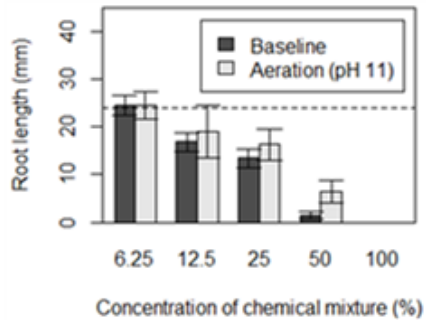
bioassay at pH adjusted back to the initial value (pH 4.6) demonstrated a significant reduction in toxicity to root. The samples filtered both at initial and high pH caused significant reductions in toxicity to shoot and seedling in comparison with the baseline. Though not significant,



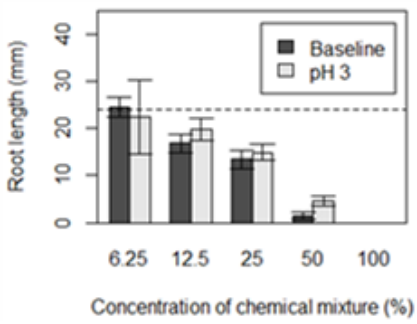
(a) EDTA treatment.



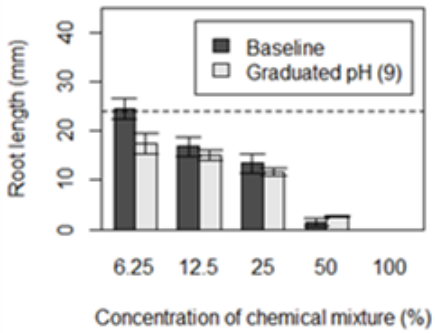
(b) C_{18} SPE at pH 9.



(c) Aeration at pH 11.



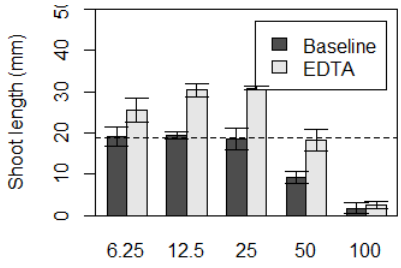
(d) pH 3 adjustment.



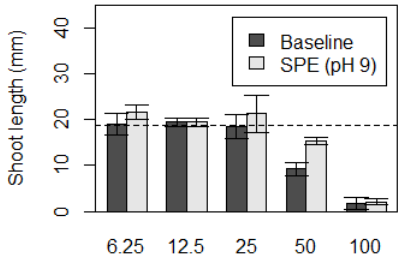
(e) Graduated pH 9.

Figure 3.7: Effect of TIE manipulations on the toxicity of chemical mixture to root length of *O. sativa* at 96-h.

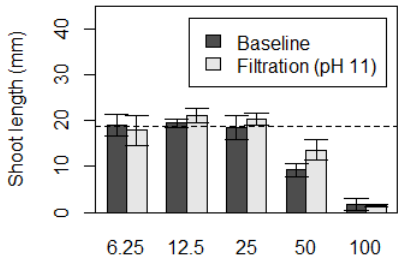
a notable increase in IC_{50} value (33.2%) was observed for filtration at pH 11 in the case of root. Similarly, IC_{50}s for the samples (at pH 9) passed through SPE column were 35.6, 64.9, and 51.6% ($p < 0.05$) for



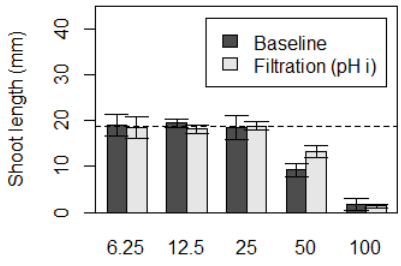
(a) EDTA treatment.



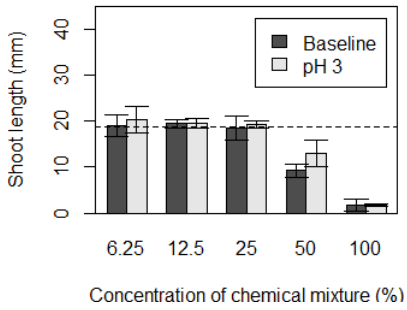
(b) C_{18} SPE at pH 9.



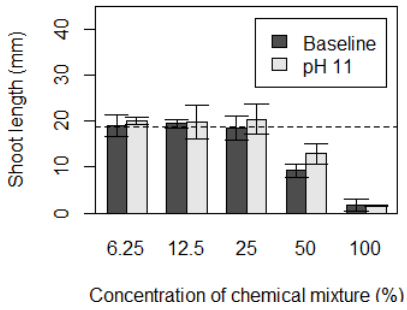
(c) Aeration at pH 11.



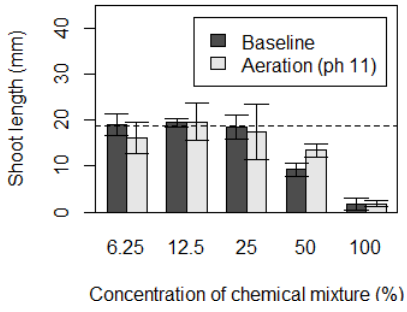
(d) Aeration at pH 11.



(e) Aeration at pH 11.



(f) pH 3 adjustment.



(g) Graduated pH 9.

Figure 3.8: Effect of TIE manipulations on the toxicity of chemical mixture to shoot length of *O. sativa* at 96-h.

root, shoot, and seedling, respectively in comparison with their baseline IC_{50}s.

The samples brought back to initial pH after aeration at pH 11 resulted in IC_{50}s of 35.9, 67.0, and 47.4% ($p < 0.05$) for root length, shoot length, and seedling length, respectively. Except for root length, no other endpoints exhibited a significant reduction in toxicity in graduated pH test.

The pH adjustment tests that followed no physical manipulations produced significantly high IC values at pH 3 and pH 11 for shoot and seedling. The only exception was root where a significantly high IC value was observed at pH 3 only.

3.4 Discussion

With the exception of methanol, none of the chemical manipulation caused significant effect on seed germination. Both IC_{25} and IC_{50} did not show any significant change in their corresponding values across the exposure durations for any of the chemical manipulations.

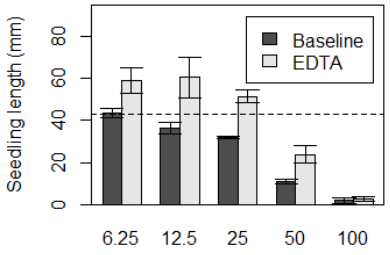
The lack of significant effect of EDTA on shoot even at 400 mg/L allows for the use of EDTA at higher concentrations in TIE. The results from the EDTA dose-response curve indicate that the EDTA can effectively be utilised in TIE at concentrations greater than those recommended by USEPA for other organisms. Thus sufficient amount of EDTA will be available in the medium to chelate cationic metals. Since plants generally exhibit metal toxicity at higher concentrations when compared with animals, a higher amount of EDTA will be required in TIE using plants

to observe any detectable change in metal phytotoxicity. Nevertheless, the use of EDTA for root length is limited to a concentration range below 200 mg/L (as it can be observed from IC values and NOECs).

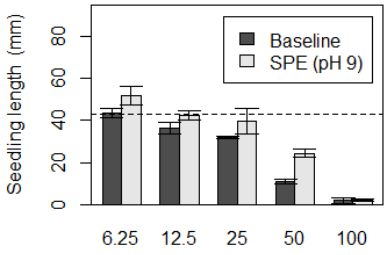
Since shoot and seedling did not show any significant hormetic response, the inclusion of these endpoints in TIE will safe guard us from reaching confounding results produced due to hormetic effects. Similar to that obtained for root, the hormetic effect of EDTA was also observed in a TIE study using marine microalga *Isochrysis galbana* (Strom et al., 2009). Conversely, hormesis was not observed in a similar study (Hogan et al., 2005) with another marine alga *Nitzschia closterium*; rather a slight inhibitory effect was observed at lower concentration (35 mg/L for 48-hr exposure). In a previous study, EDTA treatment has been found to be successful with *Lemna minor* in identifying the toxicity of copper in sewage sludge (Fjällborg and Dave, 2003).

The hardness of the water used for bioassay was found to influence the EDTA toxicity. For example, the IC_{50} (96-h) for *Ceriodaphnia dubia* was found to vary from 0.03 g/L to 0.41 g/L for very soft (10 to 13 mg/L CaCO3) and hard (160 to 180 mg/L CaCO3) reconstituted water samples, respectively (USEPA, 1991). The hardness is also found to interfere with the effectiveness of EDTA in chelating toxic metals (Fjällborg, Li, et al., 2006). Since, unlike animal tests, seed germination bioassays can be performed with pure water (without the addition of any nutrient minerals), the problems arising due to hardness interference becomes less severe in TIE using plant seeds.

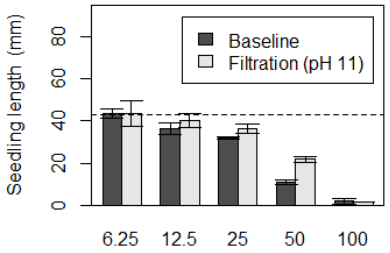
The hormetic effect of STS observed in rice root in the present study



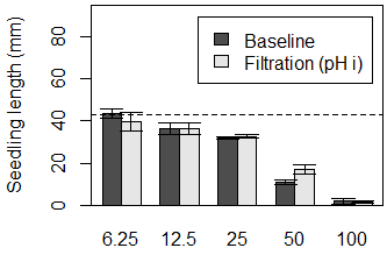
(a) EDTA treatment.



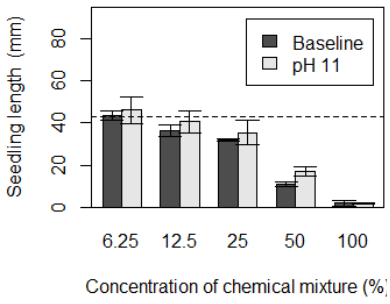
(b) C_{18} SPE at pH 9.



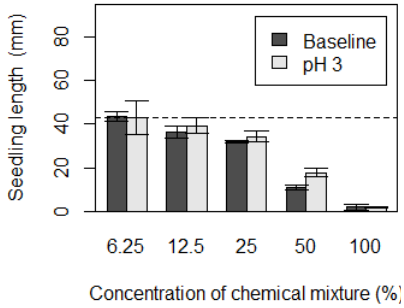
(c) Filtration at pH 11.



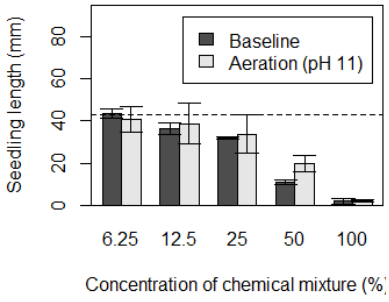
(d) Filtration at pH i.



(e) pH 11 adjustment.



(f) pH 3 adjustment.



(g) Aeration at pH 11.

Figure 3.9: Effect of TIE manipulations on the toxicity of chemical mixture to seedling length of *O. sativa* at 96-h.

is in line with that obtained for *Solanum lycopersicum* (Steinitz and Bilavendran, 2011) and for *Corymbia maculata* (Steinitz, Barr, et al., 2010). Steinitz and Bilavendran (2011) suggested that this enhancement of root growth in tomato did not reflect the correction of sulfur or sulfate nutrient deficiency in the medium. At present, no much data for the effect of STS on plants are available to explain this phenomenon and the exact role of STS in plant metabolism still remains elusive. Because the STS concentration used in this TIE study lies far above the hormetic concentration and within the CI of IC_{25}, artefacts due to hormesis will be negligible.

The NOECs obtained for shoot, seedling and seed germination in methanol is closer to that reported by Hogan et al. (2005) for a marine microalga *N. closterium*. These authors observed a significant increase in toxicity to *N. closterium* as the methanol concentration increased above 1%. Contrastingly, a significant growth enhancement (as observed in chlorophyll-a fluorescence) at or above 2% methanol was recorded for another marine microalga *Isochrysis galbana* (Strom et al., 2009). Though the present study is limited to the Phase-I characterisation and does not utilise methanol anymore in further bioassays, the methanol NOECs reported here is adequate for add-back tests performed in phase-II TIEs with *O. sativa* in future studies.

Since none of the physical and chemical manipulations involving pH change caused a significant alteration in growth response, these manipulations can successfully be used in TIEs without the need for any blank corrections. A blank correction is required when the response in the blanks

deviates markedly from that in the control. Results from both EDTA and STS show that shoot is less sensitive to chemical manipulations of TIE, and therefore shoot elongation can effectively be utilized as an endpoint in TIEs with plants.

The present toxicity characterisation study reveals the volatile, metallic, organic, and pH sensitive nature of toxicants used in the study. Reduced toxicity in EDTA treatments signifies the presence of cationic metal. Moreover, high IC values obtained for STS addition (closer to those obtained for EDTA chelation) support this further. The reduction of toxicity in the samples passed through SPE column at high pH suggests the presence of organic compound (phenol). Generally, high pH favoured the reduction of toxicity in all physical manipulations. At higher pH, metals such as Cd produces negative hydroxo complexes (Cd(OH)2)- which encourage coagulation reactions (Anielak and Schmidt, 2011). It has already been proven that mixture of cationic metals and phenol forms a non-toxic complex (Kim, Lee, et al., 2006). It is likely that such complexes form precipitates which can further be removed by filtration. This may partly explain the reduction of toxicity in the samples filtered at high pH. The complex formation at high pH may also explain the reduced toxicity at pH 9 graduation test. Reduced toxicity at elevated pH has also been observed for Cu, Fe, Mn, and Zn in a previous TIE performed with *Lactuca sativa* (Fjällborg, Li, et al., 2006).

Since the filtered samples (which already removed some toxicity) were used for SPE, greater reduction in toxicity than those observed for filtration was expected in post SPE column samples. Slightly higher IC

values for root and shoot in SPE treated samples (at pH 9) compared with those in filtered samples (pH 11) agrees with this fact. However, the reduction of toxicity in these samples may mainly be due to the adsorption of cationic metal (Cd) rather than the adsorption phenol on to the SPE column. The reason for this is that phenol produces negatively charged ions at higher pH and thus are weakly adsorbed on to the SPE column at this pH (El-Sheikh et al., 2011). SPE at pH 3 too did not result in sufficient reduction of toxicity. This might be because the volume of sample passed through the SPE column might have exceeded the breakthrough volume for phenol. The reduced toxicity in pH-adjustment samples (with no physical manipulations) shown by shoot and seedling may also be of interest. Since the pH adjustment only samples are not passed through the SPE or filter, the complex formed in the samples adjusted to extreme pH might have persisted even after returning to the initial pH. The toxicants (either already present or produced during pH shift) might have adsorbed on to this complex (even during the bioassay) rendering themselves less bioavailable (less toxic).

The reduction of toxicity observed in aerated samples at pH 11 (with back adjustment to initial pH) might probably be due to volatile loss of phenol, or be due to the complexation of phenol and Cd at high pH. The air sparging might have prevented the complex formation at lower pH, which may explain the lack of reduced toxicity at pH 3. Additional data of all TIE manipulations are given in Appendixces B1 to B9.

3.5 Conclusion

The test species *O. sativa* has successfully been employed in TIE protocol for aqueous samples. Seed germination tests are easy and cost effective as it requires no nutrient medium and no sophisticated instruments or technical knowledge in response measurement. Longer shelf life, availability at any time, and versatility in toxicity tests makes it a promising tool in ecotoxicological evaluations. Additionally, since rice seeds can be germinated in pure water without any additional substrate, the chances of artefacts created by substrate interference can be avoided. In the study presented here, the test was performed for a shorter duration (96-h) than those generally used in standard plant bioassays. Such short-term tests are useful for samples containing toxicants which are lost when kept for long durations. TIE treatments intended to reveal the toxicity of cationic metal effectively detected the presence of Cd in the chemical mixture. The presence of cationic metal was further supported by the results of STS treatment. Results from other treatments, especially C_{18} SPE, meant to reveal the presence of organics are suggestive of organic compound (phenol). Further investigations are required to evaluate the utility of *O. sativa* in TIE with environmental samples such as effluents and sediments.

Table 3.4: Impact of TIE manipulations on the toxicity of chemical mixture to root length of *Oryza sativa* at 96-h.

Manipulations	IC_{50}(%)	CI (95%)		TU	TU
		Lower	Upper		Difference[a]
Baseline	25.8	21	31	3.87	0
STS	35.4	18	52	2.83	1.04
EDTA	35.2*	28	43	2.84	1.03
Aeration (pH 3)	27.2	20	35	3.68	0.19
Aeration (pH i)	25.3	20	31	3.96	-0.09
Aeration (pH 11)	35.9*	26	46	2.79	1.08
Filtration (pH 3)	27.4	22	33	3.64	0.23
Filtration (pH i)	28.7	23	34	3.48	0.39
Filtration (pH 11)	33.2	25	41	3.01	0.86
C_{18} SPE (pH 3)	29.2	23	35	3.43	0.45
C_{18} SPE (pH i)	29.4	21	38	3.4	0.47
C_{18} SPE (pH 9)	35.6*	27	44	2.81	1.06
pH adjustment (pH 3)	34.9*	29	40	2.86	1.01
pH adjustment (pH i)	22.2	18	26	4.51	-0.64
pH adjustment (pH 11)	31.5	27	36	3.17	0.7
Graduated (pH 7)	21.7	16	27	4.61	-0.74
Graduated (pH 8)	19	8	30	5.26	-1.39
Graduated (pH 9)	31.6*	28	35	3.17	0.7

[a] = baseline TU – treatment TU; pH i = initial pH; * = IC values significantly different from baseline; CI = 95% confidence interval

Table 3.5: Impact of TIE manipulations on the toxicity of chemical mixture to shoot length of *Oryza sativa* at 96-h.

Manipulations IC_{50}(%)		CI (95%)		TU	TU
		Lower	Upper		Differencea[a]
Baseline	49.6	44	55	2.02	0
STS	56.1	49	63	1.78	0.23
EDTA	64.8*	58	72	1.54	0.47
Aeration (pH 3)	51.4	43	60	1.95	0.07
Aeration (pH i)	51	43	59	1.96	0.05
Aeration (pH 11)	67.0*	54	80	1.49	0.52
Filtration (pH 3)	54.4	49	60	1.84	0.18
Filtration (pH i)	60.2*	56	64	1.66	0.35
Filtration (pH 11)	57.3*	53	61	1.74	0.27
C_{18} SPE (pH 3)	59.2	49	70	1.69	0.33
C_{18} SPE (pH i)	56.5	51	62	1.77	0.24
C_{18} SPE (pH 9)	64.9*	59	71	1.54	0.47
pH adjustment (pH 3)	58.6*	52	65	1.71	0.31
pH adjustment (pH i)	54.9	50	60	1.82	0.2
pH adjustment (pH 11)	56.1*	52	60	1.78	0.23
Graduated (pH 7)	46.8	33	61	2.13	-0.12
Graduated (pH 8)	49.7	20	80	2.01	0
Graduated (pH 9)	54.7	47	62	1.83	0.19

[a] = baseline TU – treatment TU; pH i = initial pH; * = IC values significantly different from baseline; CI = 95% confidence interval

Table 3.6: Impact of TIE manipulations on the toxicity of chemical mixture to seedling length of *Oryza sativa* at 96-h.

Manipulations	IC_{50}(%)	CI (95%)		TU	TU
		Lower	Upper		Differencea[a]
Baseline	35.2	31	39	2.84	0
STS	47.6	34	61	2.1	0.74
EDTA	51.6*	47	56	1.94	0.9
Aeration (pH 3)	35.2	27	43	2.84	0
Aeration (pH i)	34.2	26	43	2.92	-0.08
Aeration (pH 11)	47.4*	35	59	2.11	0.73
Filtration (pH 3)	38	33	43	2.63	0.2
Filtration (pH i)	44.1*	40	48	2.27	0.57
Filtration (pH 11)	50.6*	47	55	1.98	0.86
C_{18} SPE (pH 3)	39.9	29	51	2.51	0.33
C_{18} SPE (pH i)	39.6	36	43	2.53	0.31
C_{18} SPE (pH 9)	51.6*	45	58	1.94	0.9
pH adjustment (pH 3)	44.0*	39	49	2.27	0.57
pH adjustment (pH i)	36.2	30	42	2.76	0.08
pH adjustment (pH 11)	41.9*	37	47	2.39	0.45
Graduated (pH 7)	34.4	25	44	2.91	-0.07
Graduated (pH 8)	28.9	17	41	3.46	-0.62
Graduated (pH 9)	38.3	33	44	2.61	0.23

[a] = baseline TU – treatment TU; pH i = initial pH; * = IC values significantly different from baseline; CI = 95% confidence interval

Table 3.7: Impact of TIE manipulations on the toxicity of chemical mixture to seed germination of *Oryza sativa* at 96-h.

Manipulations	LC_{50}(%)	CI (95%)		TU	TU
		Lower	Upper		Difference[a]
Baseline	59.3	48	71	1.69	0
STS	62	54	70	1.61	0.07
EDTA	61.6	44	79	1.62	0.06
Aeration (pH 3)	68.5	59	78	1.46	0.23
Aeration (pH i)	52.1	40	65	1.92	-0.23
Aeration (pH 11)	63.5	55	72	1.57	0.11
Filtration (pH 3)	64.4	51	78	1.55	0.13
Filtration (pH i)	56.5	48	65	1.77	-0.08
Filtration (pH 11)	56.1	47	65	1.78	-0.09
C_{18} SPE (pH 3)	61.7	49	75	1.62	0.07
C_{18} SPE (pH i)	60.8	51	71	1.65	0.04
C_{18} SPE (pH 9)	53.8	46	62	1.86	-0.17
pH adjustment (pH 3)	59.3	50	68	1.69	0
pH adjustment (pH i)	64.8	56	73	1.54	0.14
pH adjustment (pH 11)	59.1	46	72	1.69	-0.01
Graduated (pH 7)	61.7	49	75	1.62	0.07
Graduated (pH 8)	61.4	50	73	1.63	0.06
Graduated (pH 9)	53	44	62	1.89	-0.2

[a] = baseline TU – treatment TU; pH i = initial pH; * = IC values significantly different from baseline; CI = 95% confidence interval

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Chapter 4

The Use of *Oryza sativa* in Sediment Toxicity Assessment of the River Periyar

4.1 Introduction

Sediment plays a crucial role in maintaining the health of aquatic ecosystems (Lotufo et al., 2014; Ritter et al., 2002). Sediment functions both as a sink and as a source of contaminants (Ammar et al., 2015; Ritter et al., 2002). Anthropogenic activities lead to constant input of pollutants into the water bodies, which ultimately results in settling down of

contaminants in sediment. Sediment influences water quality by sorbing the contaminants and changing their fate and bio-availability (Go et al., 2009; Grobler et al., 1987). This may result in toxicity to aquatic organisms and thereby lead to detrimental changes in community structure (De Castro-Català et al., 2016).

Sediment risk assessment, a process aimed to evaluate the potential risks posed by sediments to ecosystems, is critical in maintaining the normal structure and functioning of the ecosystem. It was only in the late 1980s that the regulatory authorities began to show great concern about sediment risk assessment, which led to costly and extensive efforts to assess and manage several contaminated sediment sites in the western countries. Many of these sites belonged to harbours or CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act) sites (Lotufo et al., 2014). The earlier approaches to sediment quality assessments relied mainly on sediment chemistry, which was insufficient to reflect the real impact of contaminants on ecosystem (Wenning and Ingersoll, 2005). The modern approach to risk (quality or hazard) assessment for the sediment incorporates tools from several fields of science, of which toxicity tests constitute an integral part (Lotufo et al., 2014).

Sediment quality guidelines, variously called sediment quality criteria or sediment quality standards, refer to chemical-specific values of sediment-bound contaminants specified by the regulatory authorities as the legally imposed limits (Kwok, Batley, et al., 2014). Two main approaches exists for the SQGs derivation, empirical and mechanistic (Lotufo et al., 2014; Wenning and Ingersoll, 2005). The former involves the correlation of

the sediment chemistry with biological responses observed in sediment toxicity tests or in sediment community. The latter is a theoretically derived guideline which depends mainly on information from laboratory spiked sediment, water quality guidelines, and bioavailability of sediment-bound toxicants. The mechanistic approaches are mainly guided by EqP (Equilibrium Partition) theory (Ankley, Di Toro, et al., 1996; Wenning and Ingersoll, 2005). There is also a third category, consensus-based guideline, which is synthesised from both mechanistic and empirical guidelines (MacDonald et al., 2000). A detailed description on different types of sediment quality guidelines is available elsewhere (Baudo et al., 1990; Burton, 2002; Kwok, Batley, et al., 2014; Lotufo et al., 2014).

The SQGs, whether derived empirically or mechanistically, includes the following approaches: equilibrium partitioning or EqP (Di Toro et al., 1991; Kwok, Batley, et al., 2014; Wenning and Ingersoll, 2005), screening level concentration or SCL (Burnett-Seidel and Liber, 2012; Persaud et al., 1993; Von and Menzie, 2002), effects range [ERL/ERM - effects range low/effects range median according to Long and Morgan (1990)], effects level or EL (Macdonald et al., 1996; Swartz, 1999), and apparent effects threshold or AET (Barrick et al., 1988).

However, empirical and mechanistic approaches are not free from shortcomings. For example, empirically derived SQGs may sometimes wrongly assign combined effects of toxicants to a single compound. Similarly, mechanistically derived SQGs may misjudge the mixture toxicity of sediment-bound contaminants (Wenning and Ingersoll, 2005). Although a more reliable consensus-based SQG has been developed recently, it lacks

information from bioaccumulation in aquatic species (MacDonald et al., 2000).

Lack of sufficient toxicity data, especially from chronic effects, is a major hurdle in improving SQGs (MacDonald et al., 2000). Another problem encountered with the present SQGs is that the toxicity test database, which it is based on, does not cover the tropical countries well because only few sediment toxicity studies are available from these countries (Adams, Stauber, et al., 2008). Recently Kwok, Batley, et al. (2014) and Babut et al. (2005) have observed that most countries, especially tropical, owing to the paucity of sediment toxicity data, have adopted SGQs from western countries disregarding the geographical differences that may affect the valid implementation of such guidelines. Since biological response elicited by contaminants varies with geographical regions (Kwok, Leung, et al., 2007), and since the available sediment toxicity data from different geographical regions is scanty, there is an urgent need to define the cause-effect relationships between toxicants and biological responses using toxicity test data from various geographical regions (Kwok, Batley, et al., 2014).

Since species differ with regard to exposure routes and sensitivity to the same toxicant (Posthuma et al., 2002), different types of sediment toxicity tests are required to obtain a realistic picture of impacts caused by the contaminated sediments (Beketov et al., 2013; Campana et al., 2012; Sheahan and Fisher, 2012). Sediment quality assessment often involves sediment toxicity tests in which organisms are exposed (under laboratory conditions) to field-collected sediments. Sediment toxicity tests generally

include a) whole sediment toxicity tests b) sediment elutriate tests and c) sediment pore water tests. Whole sediment tests involve the simultaneous exposure of both aqueous and solid phase of the intact sediment. In elutriate tests, the organisms are exposed to the supernatant of sediment/water mixture (Ankley and Schubauer-Berigan, 1995). The pore water tests involve the exposure of organisms to water directly extracted from the space between sediment particles (Ankley and Schubauer-Berigan, 1995; EC, 1994).

Whole sediment tests have the advantage that it ensures different routes of exposure (Batley et al., 2002; Chapman et al., 2002). Elutriate tests are used to assess the impact of sediment disturbances due to resuspension and is generally associated with dredging and disposal activities (Ankley and Schubauer-Berigan, 1995). Since pore water has contaminant concentration in close equilibrium with the solid fraction and since it is required in small volume for analytical purpose (EC, 1994), pore water tests are usually preferred over other tests. Comparison between these test types has shown that bioassays using aqueous fraction of sediment are not as good as those using whole sediment (Haring et al., 2010; Harkey et al., 1994b).

While most western countries have adopted toxicity based pollution control, Asian countries including India, still lag behind in developing and implementing such guidelines. Sediment toxicity data from tropical countries will be helpful in implementing SQG locally which will also enrich the SQG database at the global level. It is surprising to note that the present scenario of sediment quality assessment in India rarely

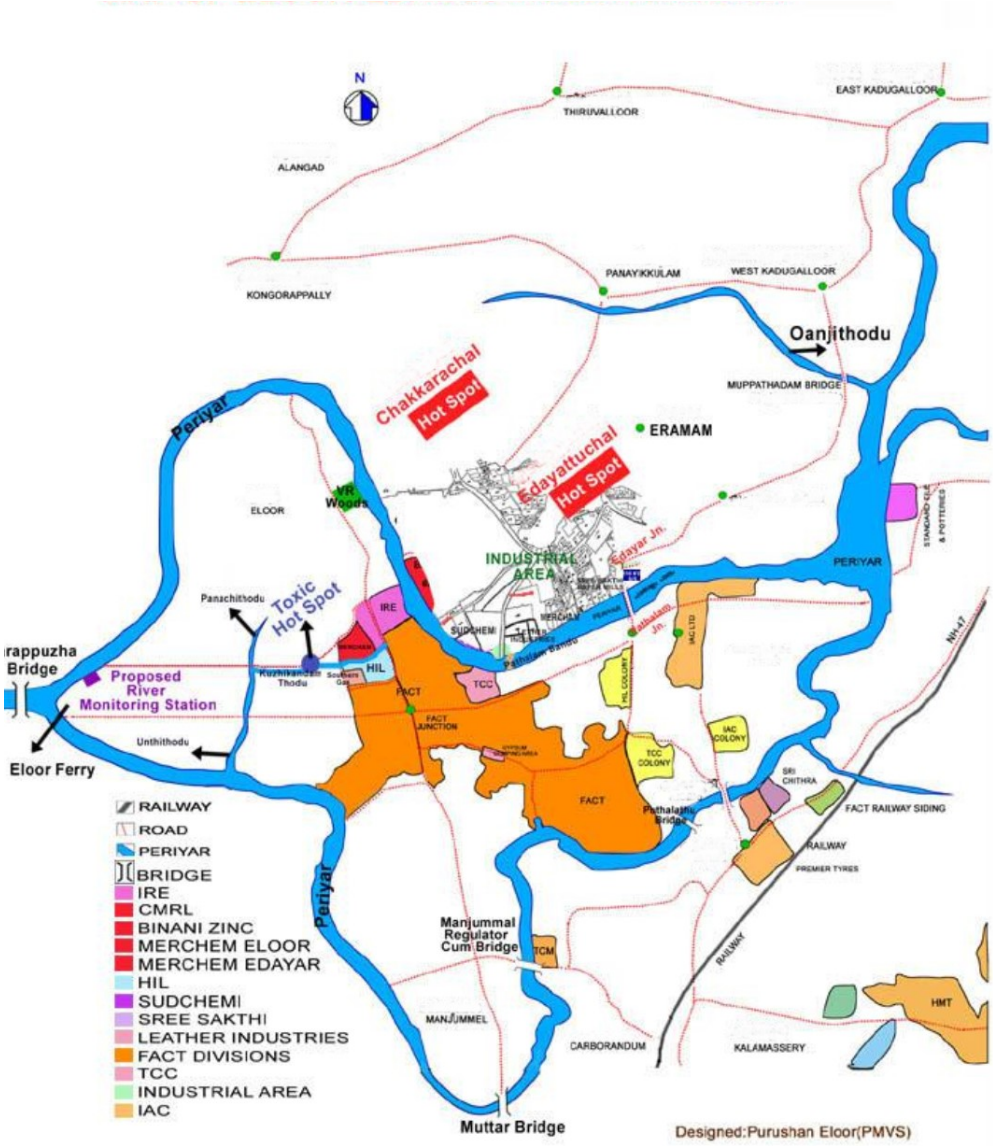


Figure 4.1: Map showing main industries loacted at Eloor-Edayar region.

involves sediment toxicity tests.

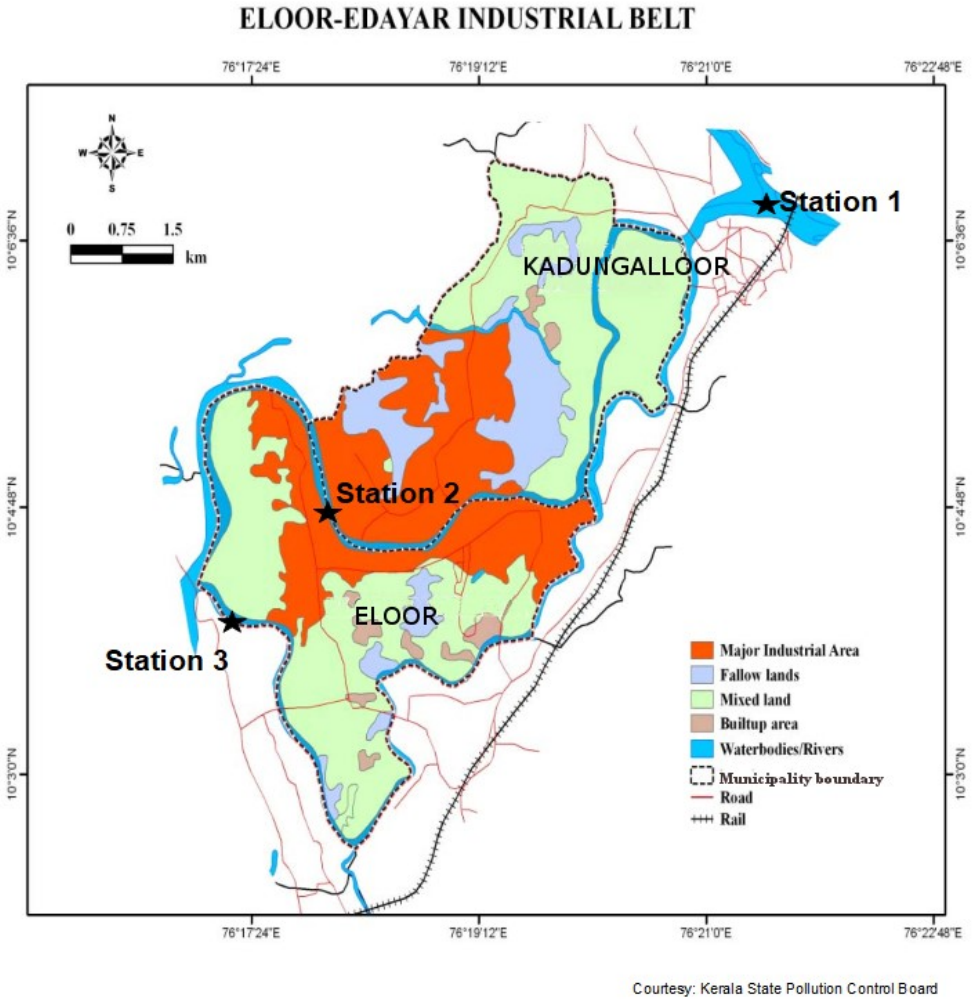


Figure 4.2: Map of the study area showing sampling stations. Station 1 represents Manappuram, Aluva (upstream of industrial belt); station 2 represents industrial belt (Eloor–Edayar region); station 3 belongs to the opening of Kuzhikkandam Thodu which receives the industrial outfall from HIL and FACT.

4.1.1 The River Periyar

The River Periyar, the longest river in Kerala, originates from the Sivagiri hills. Covering almost 244 km, it meets the Arabian sea after receiving tributaries named Perumthuraiar, Cheruthoniyar, Chittar, Perinjakkuttyar, Muthirappuzhayar, Thottiyar, and Edamalayar. At its source, the river flows through hilly areas and dense forests. At Manappuram (Aluva), the river gives off two distributaries named Mangalappuzha (north-west) which meets Chalakkudy river, and Marthandavarma (south-west), which after covering industrial belt at Eloor-Edayar region, drains into the Arabian sea. The River Periyar is the most important source of irrigation and domestic water supply in Kerala. The lower reaches of this river remains under the influence of salinity ingression, especially during low flow (Joseph, 1974). Cochin estuary, which the river bisects is one of the major Ramsar sites in the world (Dipu and Kumar, 2013). A recent study has found that the Periyar river system is the abode of several threatened fish species (Radhakrishnan and Kurup, 2010; Smakhtin et al., 2007).

Major industries in Kerala are located along the banks of the River Periyar. Of these, 90% of industries reside at the Eloor-Edayar industrial belt (Fig. 4.1). All these industries dispose their effluents into the river causing serious detrimental effects on water quality of the river. Frequent fish mortalities in this region have been a major concern for the past few decades. Several reports on the pollution status of the river indicate industries as the major source of pollution. A major portion of pollutants in the river can be attributed to acid and alkalies, radionuclides, trace

metals (Paul and Pillai, 1978), pesticides (Stringer et al., 2003; Sujatha et al., 1999), and nutrients (Martin et al., 2011). In addition, continued sand mining has been degrading the river for the past few decades (Padmalal and Maya, 2014). In one of the earlier reports on pollution status of this river, Joseph (1974) noted the absence macroinvertebrates in the industrial dense region of this river. Effect of industrial pollution on standing stock of phytoplankton has been made by Joy and Joy and Balakrishnan (1989). Further studies using effluent bioassays with algae demonstrated that the discharge in the River Periyar during dry weather is insufficient to lower the effluent concentration to a safe level (Joy, 1990; Joy and Balakrishnan, 1989). A recent study has shown that the total metal concentrations in the sediments of Cochin estuary remain higher than the mean values reported for Indian rivers (Mohan et al., 2012). Further, high Cd content in exchangeable and carbonate bound fractions of sediments has also been observed. The concentration of trace metals was found to increase in water and decrease in sediments during summer (Paul and Pillai, 1983). A detailed review of heavy metal pollution in the Cochin backwaters is available elsewhere (Anu et al., 2014). Presence of radionuclides such as thorium and uranium has also been confirmed in this river (Paul and Pillai, 1978).

The River Periyar has been one of the widely studied rivers in Kerala. Although several reports on sediment chemistry and benthic community of Cochin backwaters, including the estuarine regions of Periyar, are available, there exists no data on sediment toxicity test at present. The lack of data on sediment toxicity tests from this region creates a knowledge

gap regarding sediment database. Besides this, macrophytes are not represented well in sediment exposure studies worldwide. *Oryza sativa*, despite being a macrophyte most suitable for sediment exposure, has not been much explored for the utility in sediment toxicity tests. Perhaps the only report on the use of *O. sativa* in sediment toxicity test is by Brinke et al. (2015), in which pre-germinated (48 hours) rice seeds were used for sediment exposure. This method, however, lacks realism as it skips the early and most critical phase (seed germination) of plant growth and development.

In the present study, an effort has been made to assess the phytotoxicity of sediments from the River Periyar using *Oryza sativa*.

4.2 Materials and Methods

4.2.1 Sediment Collection

Three sampling stations (Fig. 4.2) from the lower reaches of the River Periyar were chosen for the study.

The station 1 (S1), upstream from Manappuram, Aluva, represents relatively unpolluted area (reference station). The other two stations are represented by Edayar region (S2) near FACT and the region where Kuzhikkandam thodu (S3), a canal that receives heavy loads of effluents from industries like HIL, Merchem, and FACT, opens into Muttar region of the river (further downstream from the S2). Samples were collected during monsoon (August), post-monsoon (December), and pre-monsoon (May) between 2013 and 2014. The regions covering sampling stations

received summer rains during pre-monsoon. Triplicate samples were collected using Van Veen grab sampler. The collected samples were transferred to polythene self-locking bags, transported to the laboratory and refrigerated (4°C) until use.

4.2.2 Physicochemical Analyses

All physicochemical parameters except temperature, pH, conductivity, total solids (TS), and total ammonia nitrogen (TAN) were analysed using shade-dried (under room temperature) samples after sieving through 0.64 mm sieve. Electrical conductivity (EC), pH, available potassium (K) and phosphorus (P), total organic carbon (TOC), and soil texture were analysed as per guidelines given by International Soil Reference and Information Centre (ISRIC, 2002), whereas temperature, ORP, TS, EC, and TAN were analysed as per the guidelines of EPA (Plumb, 1981). The temperature was measured using liquid-in-glass thermometer. Sediment:water mixture in the ratio of 1:5 was used for the measurement of pH, ORP, and conductivity. ORP and pH were measured using PH and ORP meters (Scientific Tech ST 2001), respectively. All quantities were expressed on a dry weight basis.

4.2.3 Whole Sediment Toxicity Test

Whole sediment bioassays were conducted in two sets, one terminated at 4th day and the other one at 7th day. Plastic cups (poly propylene) of 5 × 10 mm dimension with lids were used in the bioassay to germinate the seeds of *O. sativa* var. Jyothi. The experiment was run in triplicates with approximately 20 g of test sediment (wet weight) in each cup. Each

cup with 10 seeds of *O. sativa* was maintained under cool white light (300 µmol/m^2/s; 14:10 light:dark) at 28±2°C for 4 days or 7 days. At test termination (ie, 4th or 7th day), the plants (plantlets) were carefully removed from the cups, washed in distilled water, and photographed (camera: Kodak). The root, shoot, and seedling lengths were measured using Fiji software (Schindelin et al., 2012). Shoot length was measured from hypocotyl to the tip of the tallest leaf whereas root length was measured from hypocotyl to the root tip. In the case of post-monsoon samples (when the salinity intrusion is likely to occur), the whole sediment toxicity test was repeated with a salinity tolerant rice variety Vyttilla-6.

4.2.4 Sediment Elutriate Toxicity Test

Sediment elutriates were prepared following guidelines provided by USEPA (1998). Elutriate samples were prepared by mixing sediment and distilled water in 1:4 (v/v) ratio. The samples were placed on a rotary shaker for 30 minutes, after which the mixtures were allowed to settle down for 1 hour. The supernatant is then pipetted out and centrifuged (2000 rpm for 30 min).

For toxicity tests, a dilution series of 100, 50, 25, 12.5, 6.25, and 0% elutriate was prepared using distilled water. Triplicate of plastic Petri dishes (poly propylene; 5 × 90 mm) were then filled with 10 ml of elutriate sample. Ten rice seeds were added to each Petri dish. Petri dishes with seeds were incubated under the same conditions as those used for whole sediment tests. Root, shoot, and seedling lengths were measured after 96 hours of incubation (using Fiji software).

4.2.5 Data analysis

Data analyses were performed with R software version 3.4.0 (R Core Team, 2017). Two-way or one-way ANOVA followed by Tukey's HSD test using lsmeans package (Lenth, 2016) was performed (with p value adjustment being Tukey's) to analyse both physicochemical and biological (toxicity tests) variables of whole sediment. Correlation analyses were performed to find out the relationship between physicochemical variables as well as between physicochemical and biological variables. For elutriate tests, the IC (inhibition concentration) values were computed using drc package (Ritz et al., 2015). Only clay fractions of sediment texture were included in correlation analysis as most contaminants such as heavy metals are associated with clay fraction of sediments.

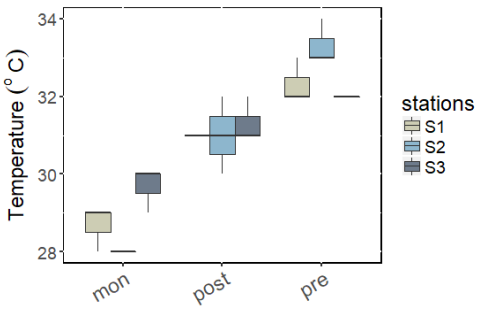
4.3 Results

4.3.1 Physicochemical Variables

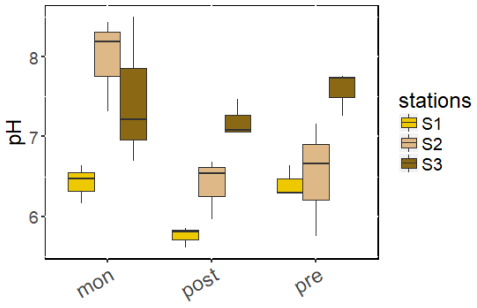
The river experienced heavy rainfall and flood during monsoon. The rainfall in post-monsoon and pre-monsoon was comparatively less compared with the monsoon. The river water turned yellowish in colour in the beginning months of monsoon due to seepage and land runoff. Frequently, the colour of the river water became reddish accompanied with fish kill.

Temperature

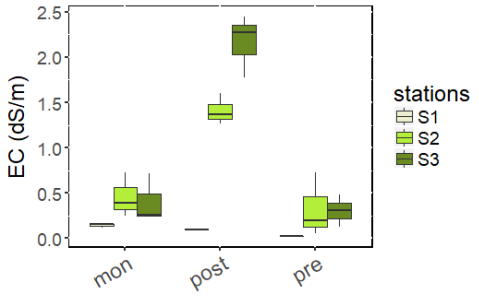
The temperature did not show the main effect of station ($p > 0.05$). The mean temperature between stations differed significantly ($p < 0.01$)



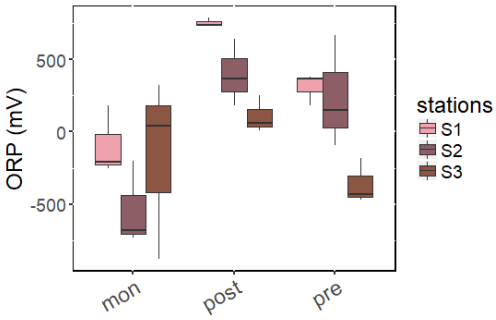
(a) Temperature.



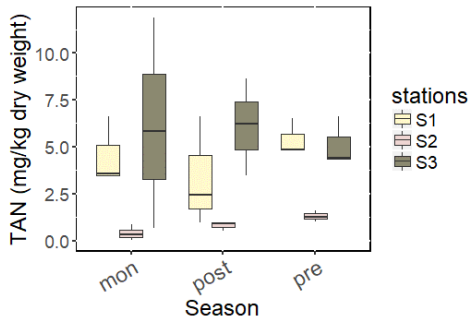
(b) pH.



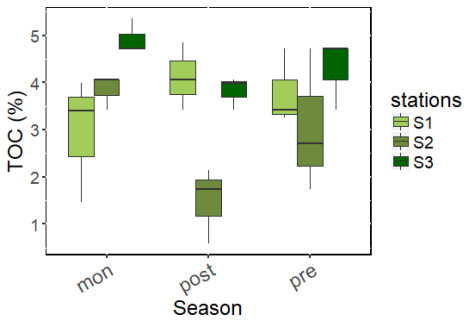
(c) Electrical conductivity.



(d) Oxidation reduction potential.

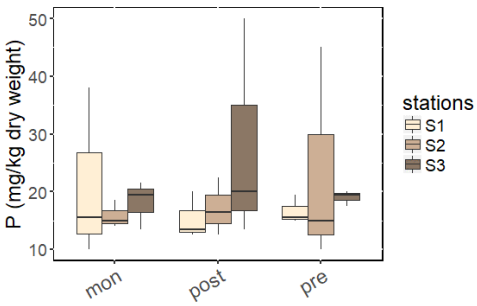


(e) Total ammonia nitrogen.

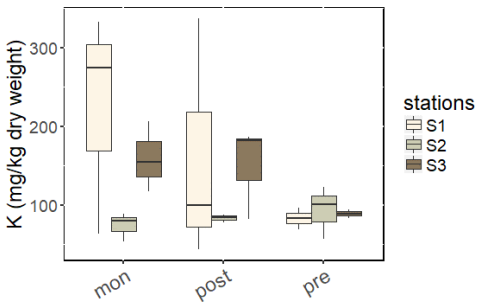


(f) Total organic carbon.

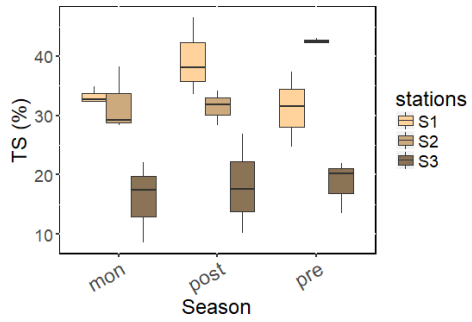
Figure 4.3: Temporal and spatial variations in physicochemical variables of sediments from the River Periyar. (***Continued on next page...***)



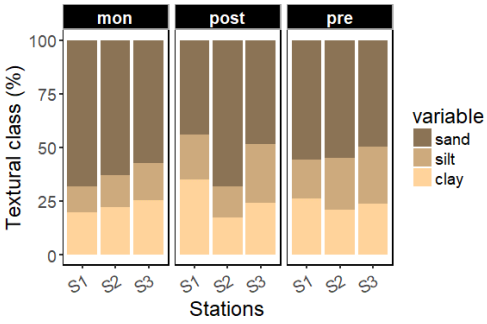
(g) phosphorus.



(h) Potassium.



(i) Total solids.



(j) Sediment texture.

Figure 4.3: (*Continued from previous page.*)

as the season changed. The mean temperature for each station was 30.7°C (SD = 1.7°C), 31°C (SD = 2.4°C), and 31°C (SD = 1°C), for station 1, station 2, and station 3, respectively (Fig. 4.3a). The highest temperature was recorded at station 2 during pre-monsoon (34°C). The mean temperature at station 2 was significantly low during monsoon (M = 28°C; $p < 0.0$) and high during pre-monsoon (M = 33.3°C; $p < 0.05$) when compared with station 3. Generally, the temperature at all the stations demonstrated a gradual increase as the pre-monsoon season approached.

pH

The pH showed both spatial ($p < 0.001$) and temporal ($p < 0.01$) changes during the period of study. No significant interaction effect was observed ($p > 0.05$). Significantly high pH values ($p < 0.05$) were recorded at station 2 and station 3 (Fig. 4.3b). Post monsoon witnessed significantly lower pH values compared with monsoon ($p < 0.01$). The sediment from station 3 maintained higher pH values (always above 7), especially during post-monsoon and pre-monsoon. In general, the station 1 always maintained a pH between 5 and 7, an ideal range for plants.

Electrical Conductivity (EC)

Both the main effects and interactions were significant for EC ($p < 0.001$). A significant peak in average EC was observed at station 3 ($M = 2.167$ dS/m, SD = 0.347 dS/m), followed by station 2 ($M = 1.413$ dS/cm, $SD = 0.169$ dS/m) during post-monsoon (Fig. 4.3c). The station 1 always recorded low mean EC ($M = 0.025$ to 0.147 dS/m). There was no significant difference in EC between any stations during monsoon and pre-monsoon.

Oxidation Reduction Potential (ORP)

For ORP, only main effects were found to be significant ($p < 0.05$). The ORP varied between -250 to 790 mV and -880 to 320 mV for station 1 and 3, respectively (Fig. 4.3d). Significantly low ORP values were recorded at station 3 compared with station 1 ($p < 0.05$). The ORP values for station 2 and 3 remained approximately the same ($p > 0.05$). The ORP recorded during post-monsoon was significantly higher than

that of monsoon ($p < 0.001$).

Total Ammonia Nitrogen (TAN)

Total ammonia did not show any significant seasonal variation ($p > 0.05$), and hence, the data were pooled across three seasons (Fig. 4.3e). No significant interaction effect between station and season was observed as well ($p > 0.05$). However, the concentration of total ammonia in the sediment varied significantly between stations ($p < 0.01$). The post hoc comparison showed that the total ammonia (pooled across seasons) at station 2 was significantly lower when compared with station 1 and station 3 ($p < 0.05$). Station 1 and station 3 did not differ significantly in total ammonia concentration. An average TAN of 0.58 mg/kg was observed at station 3. Surprisingly, lower concentrations of mean TAN were recorded at station 2 during the sampling periods ($M = 0.39$ to 1.3 mg/kg, $SD =$ 0.415 mg/L to 0.29 mg/L).

Total Organic Carbon (TOC)

The sediment TOC content demonstrated a significant variation across stations ($p < 0.01$). The interaction was also significant ($p < 0.05$). The season was not significant at all ($p > 0.05$). TOC content at station 2 showed a significant decrease during post-monsoon (Fig. 4.3f). High mean TOC content was recorded at station 3 ($M = 4.9\%$, $SD = 0.37\%$) during monsoon whereas a low value was observed at station 2 ($M =$ 1.48%, $SD = 0.81\%$) during post-monsoon. In short, the sediment TOC at station 2 and station 3 showed a characteristic increase during monsoon followed by a decrease during post-monsoon, and again an increase during

pre-monsoon.

Phosphorus (P)

There were no significant ($p > 0.05$) spatial or temporal changes in the concentration of available phosphorus during the sampling periods (Fig. 4.3). No interaction effect existed at all ($p > 0.05$). Nevertheless, the mean phosphorus content pooled across the seasons remained high at station 3 ($M = 21.6$ mg/L, $SD = 11$ mg/L) which receives an enormous amount of effluent drained by HIL through Kuzhikundam thodu.

Potassium (K)

Neither the main effects nor the interaction was significant in the case of K ($p > 0.05$). However, station 1 had a slightly higher concentration of K ($M = 224$ mg/kg, $SD = 142.1$ mg/kg) compared with station 2 during monsoon. Station 2 had more or less the same concentration of K throughout the sampling events. Though not significant, station 1 and 2 had higher values for K during monsoon and post-monsoon (Fig. 4.3g).

Total Solids (TS)

Only the main effect (station) was significant for TS ($p < 0.001$). In general, the station 3 demonstrated lower TS content ($M = 16$ to 18.6%, $SD = 6.8$ to 4.49%) throughout the sampling periods. TS content remained more or less the same at station 1 and 2 (Fig. 4.3h) during monsoon and post-monsoon. However, as the season progressed towards pre-monsoon, the TS content at station 2 peaked to a value of 42.6%.

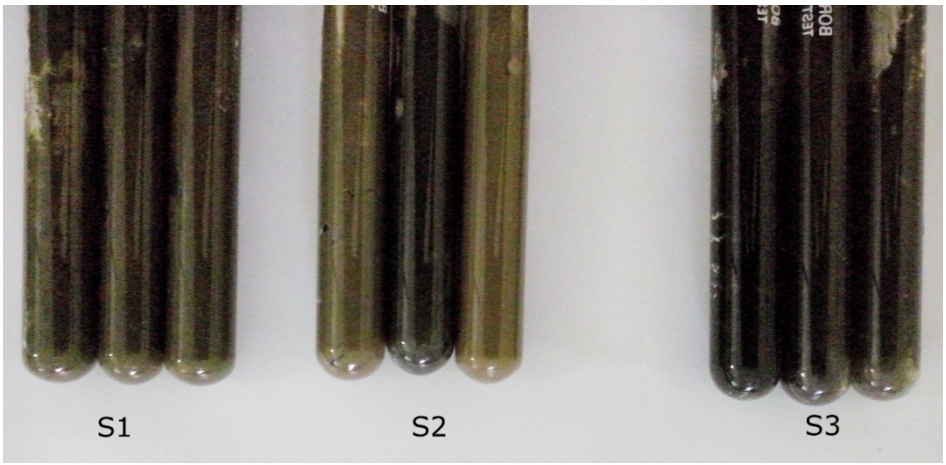


Figure 4.4: Sediment samples from Manappuram, Aluva (S1), Binanipuram (S2), and Kuzhikkandam Thodu (S3) during monsoon.

Sediment Texture

Sediment textural classes did not show any statistically significant relationship (Fig. 4.3i). The colour of the sediment samples varied between the stations. Generally, the sediment samples from station 2 imparted a characteristic yellow-brown to black colour (Fig. 4.4). Sediments from all the stations were composed mainly of sand (M = 55.9, 62.1, and 51.7% averaged across seasons for S1, S2, and S3 respectively). Though statistically not significant ($p > 0.05$), a slight reduction in the sand fraction at station 1 and 3 occurred during post-monsoon.

4.3.2 Whole Sediment Toxicity Test

All the parameters in 4-day bioassay were sensitive enough to detect the toxicity of sediment samples between stations. However, the 4-day root

elongation test was not sensitive enough to capture the interaction effect (i.e., station × season). The 4-day root elongation test showed a significant difference (Figs. 4.5 and 4.10) between stations only ($p < 0.001$). The post hoc test on root length pooled across the seasons demonstrated significantly low values ($p < 0.001$) for both station 2 ($M = 30.1$ mm, $SD = 17.87$ mm) and 3 ($M = 20.8$ mm, $SD = 15.74$ mm) compared with station 1 ($M = 74.6$ mm, $SD = 15.72$ mm). However, the root length for stations 2 and 3 did not differ significantly from each other ($p > 0.05$).

Unlike 4-day root elongation, the 4-day shoot elongation detected a significant difference between stations ($p < 0.001$) as well as seasons ($p < 0.05$). The shoot in 4–day test was significantly shorter in sediment samples from station 2 and 3 in relation to reference station (station 1). Shoot lengths for station 2 and 3 were two-fold lower (relative to station 1) with a mean value of 15.7 mm ($SD = 8.5$ mm), and 13.4 mm ($SD = 8.5$ mm), respectively. No significant difference in shoot length could be detected between the samples from station 2 and 3. The seasonal difference was also significant with a slight decrease in shoot length during post-monsoon compared with monsoon ($p < 0.05$).

The 4-day seedling growth demonstrated a pattern similar to that of the root with station being the only significant factor ($p < 0.001$). Both station 2 and 3 were characterised by decreased seedling length ($p < 0.001$). This decrease was prominent (when compared with station 1) with a 30.3% reduction for station 3 followed by 40.7% for station 2.

Only one main effect (station) was significant ($p < 0.001$) in the case of the 7-day test for root and seedling (Fig. 4.6). Shoot, however, showed

a significant effect of both station ($p < 0.001$) and season ($p < 0.01$). The root length for station 1 remained more or less the same (Fig. 4.11) during all seasons. The root length (pooled across seasons) turned to be less than 50% for both station 2 ($M = 51.9$ mm, $SD = 11.8$ mm) and 3 ($M = 48.9$ mm, $SD = 33.9$ mm) compared with station 1 ($M = 112.6$, $SD = 11.3$, $p < 0.01$). However, no significant difference could be observed between station 2 and 3 ($p > 0.05$). The shoot length reached around 57% and 31% for station 2 and 3, respectively, compared with station 1 ($M = 80.57$, $SD = 8.01$, $p < 0.01$). Shoot growth was significantly reduced during post-monsoon reaching only about 70% of the value obtained for monsoon ($p < 0.05$).

As in the case of root length, the seedling length on the 7th day showed a significant difference between stations only ($p < 0.001$). There was approximately a two-fold decrease in seedling length for station 2 ($M = 98.2$, $SD = 18.5$) and 3 ($M = 100.3$, $SD = 53.9$) on 7th day compared with station 1 ($M = 203.2$, $SD = 18.5$, $p < 0.01$). There was a slight, but non-significant increase in the seedling length in samples from station 1 during pre-monsoon.

Whole sediment toxicity test was repeated for post-monsoon samples using Vyttila-6 as the test species. Toxicity test (4-day) with Vyttila-6 showed a significantly shorter root, shoot, and seedling length (Figs. 4.7a - 4.7c) for station 2 and 3 in relation to station 1 ($p < 0.01$). All the morphometric variables for station 3 produced significantly lower values than those for station 2 on the 4th day. However, in the case of 7-day test, the toxic effect of sediment was found to be more or less the same

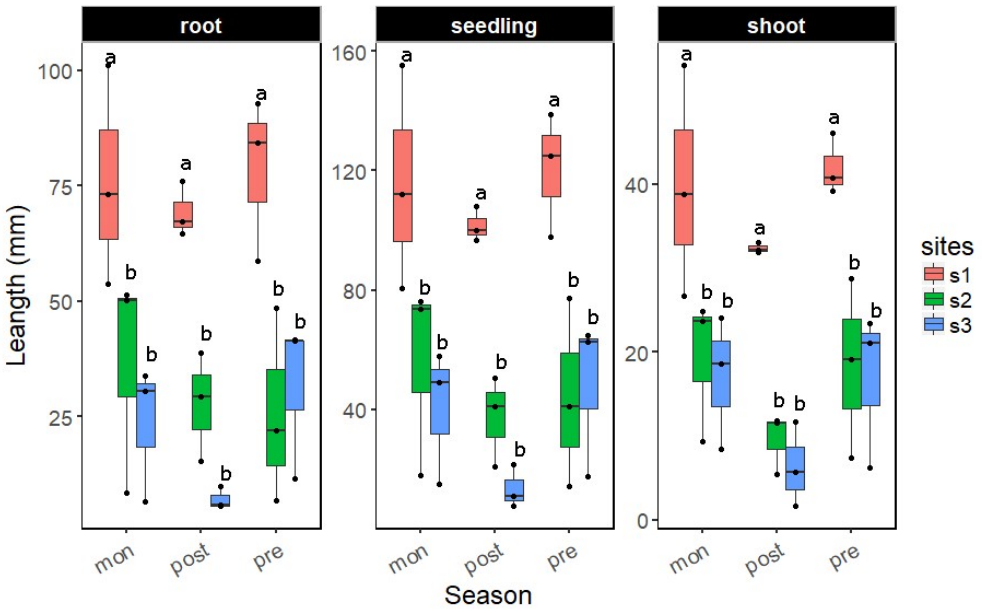


Figure 4.5: Temporal and spatial variations in biological variables of *O. sativa* exposed to contaminated sediments from the River Periyar for 4 days. Boxes with a common letter do not differ significantly ($p > 0.05$) between sites as analysed by Tukey's HSD test.

for station 2 and 3 (Figs. 4.8 and 4.12), which is evident from the lack of significant difference between station 2 and 3 in any of the morphometric parameters ($p > 0.05$). Both station 2 and 3 remained significantly lower than station 1 with regard to root, shoot, and seedling length on the 7th day.

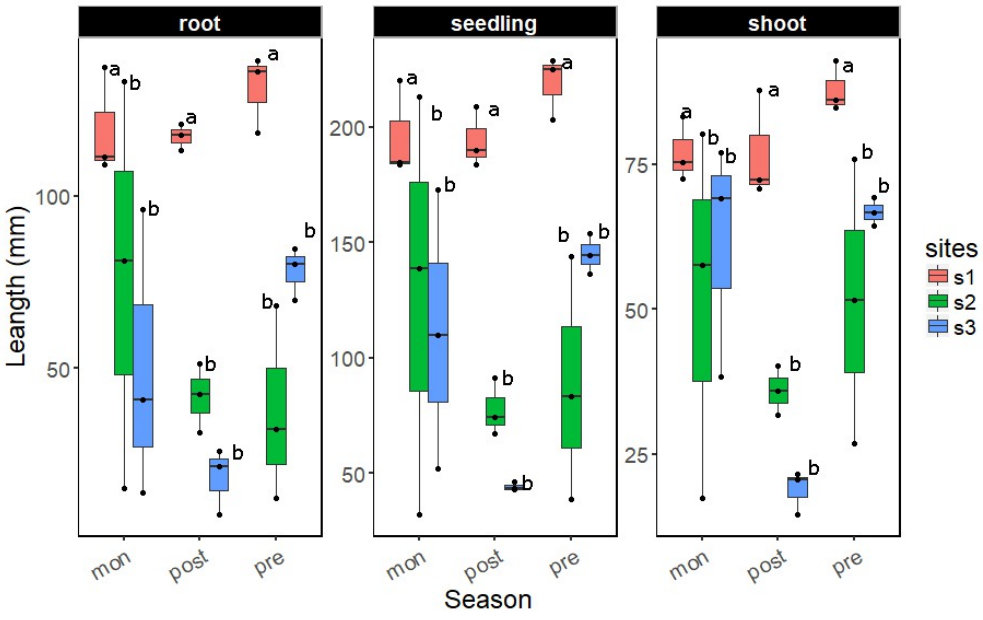
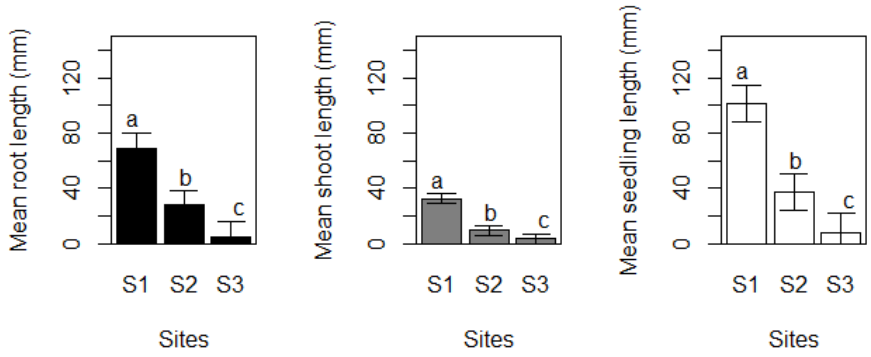


Figure 4.6: Temporal and spatial variations in biological variables of *O. sativa* exposed to contaminated sediments from the River Periyar for 7 days. Boxes with a common letter do not differ significantly ($p > 0.05$) between sites as analysed by Tukey's HSD test.

4.3.3 Sediment Elutriate Test

Except for station 2, there was no significant dose-response relationship in any of the elutriate sample. Root growth was the only variable which was significantly affected by the elutriate exposure. An increase in elutriate concentration severely inhibited the root growth at station 2. Though statistically not significant, a decreasing trend in root growth towards the top concentration in station 3 elutriate was also observed (Fig. 4.9). The calculated EC_x values were highly variable as evidenced by wider



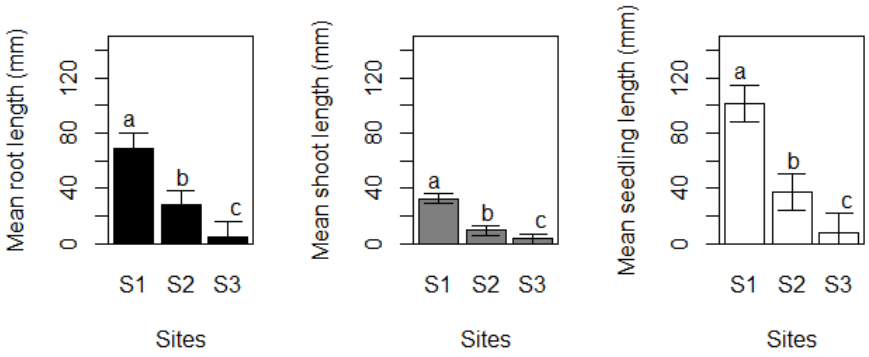
(a) Monsoon. **(b)** Post-monsoon. **(c)** Pre-monsoon.

Figure 4.7: Root (a), shoot (b), and seedling (c) lengths of salt tolerant varaitey (Vyttila-6) of *O. sativa* in sediment (post-monsoon) exposure of 4 days. Bars (confidence intervals) with a common letter do not differ significantly ($p > 0.05$) as analysed by Tukey's HSD.

Table 4.1: Inhibition concentrations (%) and 95% Confidence Interval for elutriate sample from station 2.

Endpoint	Estimate	Lower	Upper
$\mathbf{IC_{10}}$	13	1.94	87.08
$\mathbf{IC_{25}}$	27.9	7.89	98.48
$\mathbf{IC_{50}}$	59.7	27.05	131.72

confidence intervals (Table 4.1).



(a) Monsoon. **(b)** Post-monsoon. **(c)** Pre-monsoon.

Figure 4.8: Root (a), shoot (b), and seedling (c) lengths of salt tolerant varaitey (Vyttila-6) of *O. sativa* in sediment (post-monsoon) exposure of 7 days. Bars (confidence intervals) with a common letter do not differ significantly ($p > 0.05$) as analysed by Tukey's HSD.

4.3.4 Correlation Study

A significant negative correlation between pH and ORP was observed at all the three stations. (Table. 4.2 – 4.4). The TAN demonstrated a negative correlation with TS at station 1, positive correlation with temperature at station 2, and positive and negative correlations with pH and ORP, respectively at station 3. A strong negative correlation between TOC and ORP was observed at station 2. Except for station 1, where a negative correlation between P and TOC existed, no other stations showed a significant relationship between P and other variables.

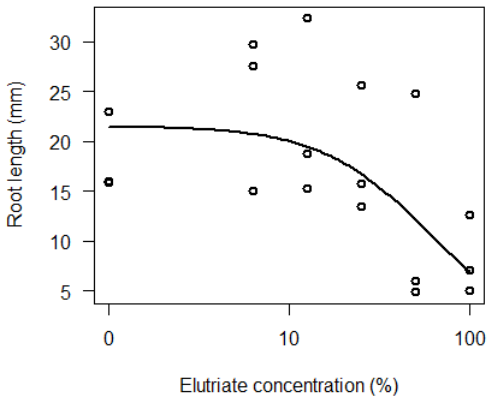


Figure 4.9: Dose-response relationship between root growth of *O. sativa* and sediment elutriate from station 2.

A significant negative correlation between TOC and EC was observed at station 2.

Shoot length was positively correlated ($r = 0.76$, $p < 0.05$) with TAN in the 7-day exposure of station 1 (Table 4.5). The station 2 demonstrated a positive, but non-significant ($r = 0.21,\ 0.25$; $p > 0.05$) relationship between TAN and shoot growth both at day 4 and 7 (Table 4.6). Station 2 completely lacked any significant correlation between physicochemical and morphometric variables. On the contrary, all the growth parameters were negatively correlated with the TAN at station 3 (Table 4.7); however, this relationship was not significant ($p > 0.05$). The EC showed a significant negative correlation with all the growth parameters in 4-day and 7-day tests at station 3; the relationship was much stronger for the 7-day shoot length. For additional details of statistics, see Appendices C1 and C2 for physicochemical variables, Appendices C3 and C4 for biological variables.

4.4 Discussion

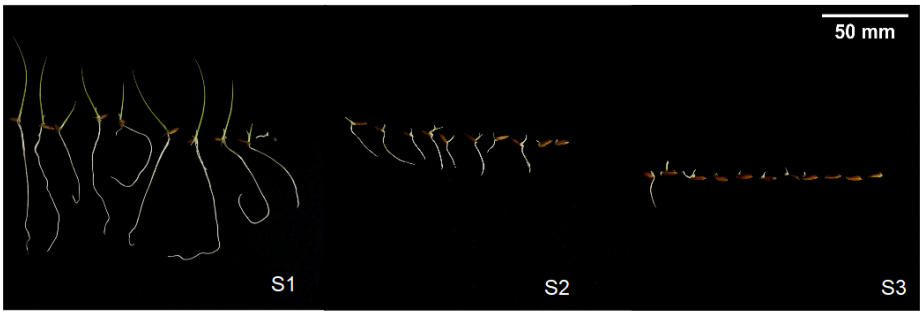
The pattern of seasonal variation in temperature observed in the study is in agreement with those observed by Joy and Balakrishnan (1989) with water samples collected from Eloor region (the lowest during monsoon and highest during pre-monsoon). The temperature profile of station 2 strengthens the findings of these authors, who observed an increase in temperature of river water towards Edayar (closer to station 2) in pre-monsoon, which could be attributed to the heavy discharge of heated effluents from the factories nearby (Binani Zinc Ltd., FACT and TCC). A significant increase in the sediment temperature can affect the mineralisation of organic matter, phosphorus and nitrogen by influencing the metabolic rates of sediment-dwelling microbes (Sanz-Lázaro et al., 2015). The increase in sediment temperature may also enhance the release of inorganic nutrients to the water column. This leads to algal blooms. Organic pollution under elevated temperature may stimulate anaerobic respiration in sediments (Sanz-Lázaro et al., 2015). Further, some pesticides show increased toxicity at elevated temperature (Lydy et al., 1990). The ammonia, which is in close relationship with pH, might have also been converted to its unionised form at high pH resulting from the increase in temperature.

The lower concentrations of TAN observed at station 2 might be due to higher pH recorded in this region especially during monsoon. The negative correlation between TAN and pH supports this further. The ionic strength, pH, and temperature influence the speciation of ammonia in

the aqueous medium (Emerson et al., 1975), which makes this compound a special concern in sediment monitoring. The ammonia at its pKa (pH 9.25) dissociates into equal amounts of unionised and unionised forms. A one-unit increase above this will change the concentration of unionised ammonia to approximately 90% (USEPA, 1991). At elevated pH, a major portion of TAN exists as NH_3 (unionised ammonia, which is more volatile than its ionised form), which is subsequently lost through the exchange from sediment to the water column. The unionised ammonia is more toxic than its ionised counterpart (USEPA, 1991) and it can pass through the cell membrane easily. It is also known to inhibit the exogenous respiration of citrate, glucose, and malate in plants (Vines and Wedding, 1960).

Previous studies have reported higher concentrations of ammonia in water samples from station 2 (Devi et al., 1991; Joy and Balakrishnan, 1989). These reports point towards the industrial outfalls as the cause of higher ammonia concentration in this area. In the present study, however, the total ammonia concentration in sediment from station 2 was low when compared with other two stations, which was against the observations made with water samples in previous studies. This low amount of total ammonia in sediments at station 2, despite the close vicinity of industries, could also be explained by the low total organic carbon (TOC) content at station 2 compared with stations 1 and 3. Organic matter forms the main substrate for mineralisation and subsequent increase in TAN content in pore water (Frazier et al., 1996). When TOC is low, the ammonia from sandy sediments will be fluxed to surface water where it results in high total ammonia (Frazier et al., 1996).

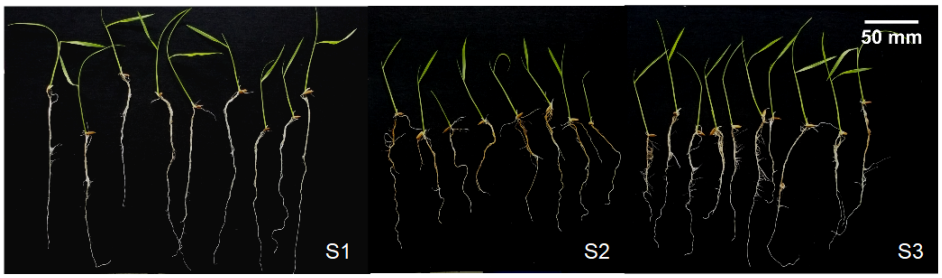
(a) Monsoon.



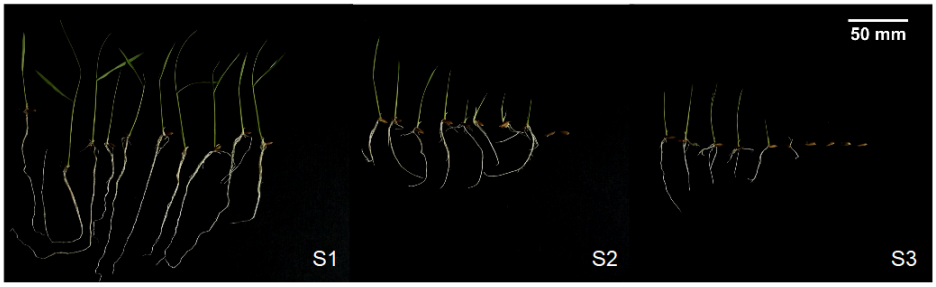
(b) Post-monsoon.

(c) Pre-monsoon.

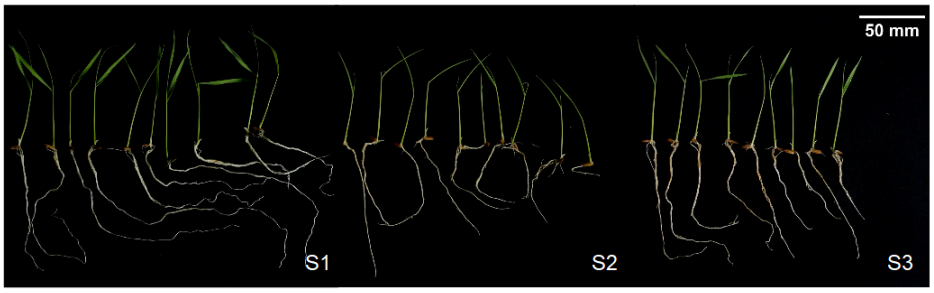
Figure 4.10: *O. sativa* seedlings after 4 days of exposure to sediments sediment collected during 3 seasons from the River Periyar. S1, S2, and S3 denote station 1, station 2, and station 3, respectively.



(a) Monsoon.

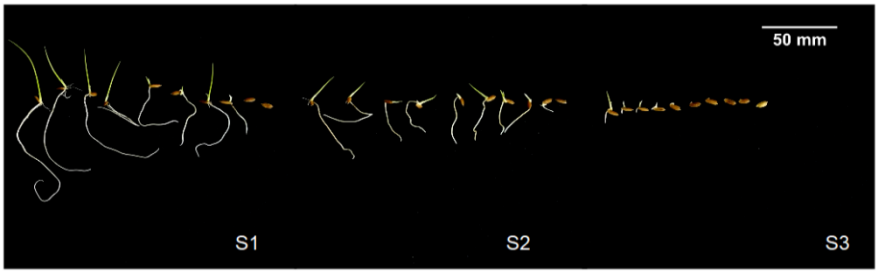


(b) Post-monsoon.

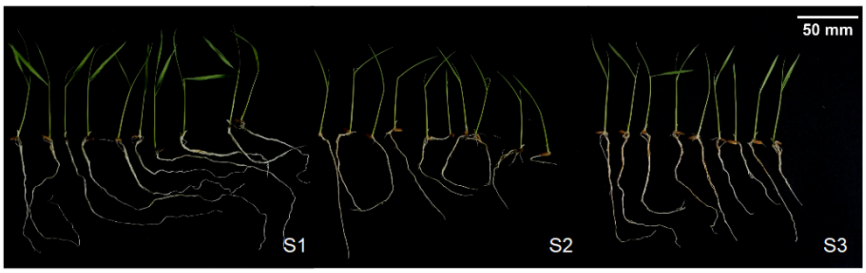


(c) Pre-monsoon.

Figure 4.11: *O. sativa* seedlings after 7 days of exposure to sediments sediment collected during 3 seasons from the River Periyar. S1, S2, and S3 denote station 1, station 2, and station 3, respectively.



(a) *O. sativa* seedlings after 4 days of exposure.



(b) *O. sativa* seedlings after 7 days of exposure.

Figure 4.12: *O. sativa* seedlings after 4 (a) and 7 (b) days of exposure to sediment collected during post-monsoon from the River Periyar. S1, S2, and S3 denote station 1, station 2, and station 3, respectively.

Table 4.2: Pearson's correlation matrix for physicochemical variables of sediment from station 1 (S1). Bold figures represent p -values. Asterisks denote significant relationships.

VARIABLES	Temperature	TS	TAN	pH	ORP	EC	P	K	Clay	TOC
Temperature	1									
TS	-0.040	1.000								
	0.924									
TAN	0.130	-0.710	1.000							
	0.734	**0.033***								
pH	-0.150	-0.630	0.460	1.000						
	0.707	**0.069**	**0.208**							
ORP	0.480	0.430	-0.300	-0.710	1.000					
	0.196	**0.250**	**0.427**	**0.033***						
EC	-0.920	0.180	-0.150	-0.030	-0.290	1.000				
	0.000*	**0.650**	**0.695**	**0.946**	**0.449**					
P	-0.420	-0.250	-0.240	0.380	-0.020	0.290	1.000			
	0.255	**0.509**	**0.532**	**0.316**	**0.965**	**0.452**				
K	-0.370	0.480	-0.210	-0.240	-0.340	0.390	-0.510	1.000		
	0.321	**0.192**	**0.584**	**0.531**	**0.364**	**0.301**	**0.163**			
Clay	0.410	0.300	-0.240	-0.490	0.350	-0.130	-0.460	0.140	1.000	
	0.273	**0.439**	**0.531**	**0.177**	**0.352**	**0.733**	**0.208**	**0.713**		
TOC	0.480	-0.030	0.410	-0.510	0.310	-0.340	-0.780	0.220	0.460	1.000
	0.194	**0.933**	**0.278**	**0.158**	**0.410**	**0.366**	**0.012***	**0.564**	**0.213**	

Table 4.3: Pearson's correlation matrix for physicochemical variables of sediment from station 2 (S2). Bold figures represent p -values. Asterisks denote significant relationships.

VARIABLES	Temperature	TS	TAN	pH	ORP	EC	P	K	Clay	TOC
Temperature	1									
TS	0.630	1.000								
	0.069									
TAN	0.810	0.490	1.000							
	0.008*	**0.182**								
pH	-0.740	-0.440	-0.530	1.000						
	0.023*	**0.232**	**0.141**							
ORP	0.700	0.390	0.490	-0.990	1.000					
	0.035*	**0.304**	**0.184**	**0.00***						
EC	-0.020	-0.380	-0.050	-0.530	0.580	1.000				
	0.967	**0.310**	**0.908**	**0.142**	**0.100**					
P	0.280	0.320	0.260	-0.140	0.100	-0.160	1.000			
	0.464	**0.400**	**0.505**	**0.724**	**0.805**	**0.679**				
K	0.310	0.440	0.110	-0.160	0.100	-0.210	0.630	1.000		
	0.409	**0.238**	**0.771**	**0.683**	**0.795**	**0.595**	**0.071**			
Clay	-0.030	0.410	-0.360	-0.110	0.040	-0.100	0.070	0.440	1.000	
	0.946	**0.269**	**0.337**	**0.777**	**0.911**	**0.808**	**0.849**	**0.238**		
TOC	-0.330	0.180	-0.170	0.650	-0.710	-0.690	0.530	0.360	0.260	1.000
	0.387	**0.647**	**0.654**	**0.059**	**0.033***	**0.040***	**0.145**	**0.342**	**0.507**	

Table 4.4: Pearson's correlation matrix for physicochemical variables of sediment from station 3 (S3). Bold figures represent p -values. Asterisks denote significant relationships.

VARIABLES	Temperature	TS	TAN	pH	ORP	EC	P	K	Clay	TOC
Temperature	1.000									
TS	0.060	1.000								
	0.882									
TAN	-0.010	-0.300	1.000							
	0.980	**0.430**								
pH	0.040	-0.620	0.680	1.000						
	0.918	**0.076**	**0.045***							
ORP	-0.170	0.370	-0.710	-0.900	1.000					
	0.661	**0.333**	**0.031***	**0.001***						
EC	0.230	0.080	0.260	-0.240	0.350	1.000				
	0.557	**0.840**	**0.495**	**0.527**	**0.361**					
P	0.010	-0.540	-0.130	0.170	0.240	0.260	1.000			
	0.979	**0.137**	**0.730**	**0.657**	**0.527**	**0.502**				
K	-0.400	0.410	-0.080	-0.660	0.510	0.340	-0.490	1.000		
	0.280	**0.274**	**0.839**	**0.056**	**0.165**	**0.372**	**0.179**			
Clay	-0.100	0.860	-0.460	-0.770	0.550	-0.020	-0.470	0.580	1.000	
	0.792	**0.003***	**0.218**	**0.015***	**0.128**	**0.955**	**0.206**	**0.104**		
TOC	-0.440	-0.200	0.390	0.260	-0.420	-0.410	-0.490	0.160	-0.170	1.000
	0.238	**0.607**	**0.301**	**0.505**	**0.263**	**0.270**	**0.183**	**0.678**	**0.667**	

Table 4.5: Pearson's correlation matrix for physicochemical variables of sediment from station 1 (S1) and morphometric variables of *O. sativa*. Bold figures represent p -values. Asterisks denote significant relationships.

Variable	Duration	Temperature	TS	TAN	pH	ORP	EC	P	K	Clay	TOC
Root	4-day	0.070	0.030	-0.310	0.110	-0.260	-0.260	0.050	-0.150	-0.310	-0.390
		0.858	**0.946**	**0.418**	**0.775**	**0.506**	**0.505**	**0.895**	**0.702**	**0.416**	**0.301**
Root	7-day	0.450	0.020	0.490	0.390	-0.180	-0.420	-0.500	0.060	0.020	0.190
		0.222	**0.962**	**0.185**	**0.299**	**0.639**	**0.261**	**0.170**	**0.873**	**0.966**	**0.630**
Shoot	4-day	0.020	-0.310	-0.100	0.260	-0.410	-0.330	0.150	-0.140	-0.480	-0.280
		0.952	**0.419**	**0.797**	**0.494**	**0.277**	**0.381**	**0.706**	**0.728**	**0.189**	**0.468**
Shoot	7-day	0.600	-0.470	0.760	0.420	-0.140	-0.600	-0.440	-0.230	-0.130	0.400
		0.089	**0.207**	**0.016***	**0.263**	**0.726**	**0.089**	**0.233**	**0.559**	**0.743**	**0.283**
Seedling	4-day	0.060	-0.090	-0.240	0.170	-0.320	-0.290	0.090	-0.150	-0.380	-0.360
		0.887	**0.812**	**0.526**	**0.663**	**0.404**	**0.445**	**0.823**	**0.702**	**0.310**	**0.339**
Seedling	7-day	0.550	-0.190	0.640	0.430	-0.180	-0.530	-0.510	-0.060	-0.040	0.290
		0.128	**0.625**	**0.063**	**0.249**	**0.652**	**0.146**	**0.161**	**0.882**	**0.909**	**0.444**

Table 4.6: Pearson's correlation matrix for physicochemical variables of sediment from station 2 (S2) and morphometric variables of *O. sativa*. Bold figures represent p -values. Asterisks denote significant relationships.

Variable	Duration	Temperature	TS	TAN	pH	ORP	EC	P	K	Clay	TOC
Root	4-day	-0.210	-0.500	0.040	0.400	-0.410	-0.230	0.410	-0.040	-0.530	0.360
		0.597	**0.169**	**0.921**	**0.285**	**0.274**	**0.550**	**0.270**	**0.910**	**0.141**	**0.347**
Root	7-day	-0.420	-0.570	-0.200	0.620	-0.580	-0.320	0.160	-0.330	-0.630	0.330
		0.256	**0.111**	**0.611**	**0.075**	**0.101**	**0.408**	**0.677**	**0.382**	**0.070**	**0.379**
Shoot	4-day	-0.040	-0.020	0.210	0.330	-0.370	-0.570	0.510	0.020	-0.330	0.570
		0.928	**0.968**	**0.579**	**0.392**	**0.328**	**0.106**	**0.159**	**0.963**	**0.389**	**0.110**
Shoot	7-day	-0.020	-0.140	0.250	0.350	-0.340	-0.450	0.470	-0.140	-0.650	0.420
		0.955	**0.716**	**0.520**	**0.358**	**0.369**	**0.230**	**0.206**	**0.726**	**0.060**	**0.263**
Seedling	4-day	-0.160	-0.360	0.100	0.390	-0.410	-0.350	0.460	-0.030	-0.480	0.440
		0.688	**0.344**	**0.800**	**0.298**	**0.271**	**0.350**	**0.211**	**0.949**	**0.187**	**0.235**
Seedling	7-day	-0.290	-0.430	-0.040	0.540	-0.510	-0.380	0.280	-0.270	-0.660	0.380
		0.453	**0.252**	**0.925**	**0.134**	**0.160**	**0.320**	**0.463**	**0.482**	**0.055**	**0.317**

Table 4.7: Pearson's correlation matrix for physicochemical variables of sediment from station 3 (S3) and morphometric variables of *O. sativa*. Bold figures represent p -values. Asterisks denote significant relationships.

Variable	Duration	Temperature	TS	TAN	pH	ORP	EC	P	K	Clay	TOC
Root	4-day	-0.030	0.510	-0.530	-0.250	0.070	-0.700	-0.350	-0.090	0.640	0.010
		0.941	**0.165**	**0.138**	**0.511**	**0.864**	**0.035***	**0.355**	**0.818**	**0.064**	**0.984**
Root	7-day	0.210	0.260	-0.580	-0.250	0.000	-0.720	-0.490	-0.040	0.330	0.100
		0.580	**0.500**	**0.103**	**0.510**	**0.998**	**0.028***	**0.180**	**0.909**	**0.384**	**0.791**
Shoot	4-day	-0.300	0.560	-0.480	-0.240	0.100	-0.670	-0.350	0.000	0.630	0.130
		0.431	**0.119**	**0.193**	**0.532**	**0.801**	**0.047***	**0.350**	**0.998**	**0.066**	**0.739**
Shoot	7-day	-0.140	0.200	-0.400	-0.080	-0.140	-0.920	-0.480	-0.080	0.330	0.380
		0.717	**0.609**	**0.280**	**0.830**	**0.728**	**0.000***	**0.195**	**0.838**	**0.381**	**0.318**
Seedling	4-day	-0.130	0.530	-0.520	-0.250	0.080	-0.700	-0.360	-0.060	0.650	0.050
		0.746	**0.141**	**0.149**	**0.512**	**0.839**	**0.035***	**0.346**	**0.880**	**0.060**	**0.896**
Seedling	7-day	0.070	0.190	-0.590	-0.190	-0.010	-0.840	-0.400	-0.120	0.310	0.170
		0.860	**0.616**	**0.096**	**0.617**	**0.973**	**0.005***	**0.283**	**0.767**	**0.419**	**0.661**

As opposed to generalisations, the positive correlation between TAN and pH at station 3 remains complex for interpretation. However, it should be noted that this station receives a constant input of effluents from a pesticide factory and hence, these results should be interpreted in the light of degradation aspects of pesticides. Tomizawa (1975) has shown that the reducing condition can greatly enhance the degradation of organophosphorus pesticides containing substituents like nitro groups. Jones and Hood (1980) found that the degradation products of pesticides in estuarine sediments, especially under low O_2 levels, can significantly inhibit the ammonium oxidation. It should also be noted that pesticides, especially organophosphates degrade under elevated pH (Singh and Walker, 2006; Singh, Walker, et al., 2003). Thus, the degradation of pesticides at elevated pH accompanied by the lack of ammonium oxidation might have caused the increase in total ammonia in sediments at the station 3 which may explain the positive correlation between pH and TAN in this region. Though the pH at station 2 and station 3 was comparatively higher in the present study, it always remained below 9 (i.e., pKa of ammonia) and hence, the production of unionised ammonia was limited to some extent.

The positive correlation between pH and TAN could also be a result of the artefact created by the pH decrease accompanied with the ammonia adsorption by the sediment during the storage period between the quantification of ammonia and the initial pH measurements. Due to increased turbulence in the upstream region, a major portion of TAN at station 1 might have diffused into the overlying water. Since the diffusion

rate of ammonia gas is a function of sediment water content (Zhong et al., 2015), the high TS (i.e., low water content) might have led to its negative correlation with TAN. The positive correlation between TAN and shoot length for stations 1 and 2 is because the plants can metabolise ammonium to a certain extent. Besides this, shoot of rice has been shown to be less sensitive to ammonia (Qi et al., 2012).

The lower pH values for Eloor region during pre-monsoon are in agreement with results obtained for water samples in previous studies (Joy and Balakrishnan, 1989; KSTSCE, 2009). This lowering of pH could be attributed to the effluent release by industries in the vicinity of this region. The pH influences the soil nutrient availability and heavy metal toxicity. Generally, plants do not prefer soils with extremely high or low pH. The low pH mobilises metals like Cu, Zn, Pb, and Al (Delhaize and Ryan, 1995; Reddy et al., 1995) whereas, the high pH stimulates the release of phosphorous in the sediment water interface (Li et al., 2013) which may, in turn, cause eutrophication. The pH at 4.0 and 10 has been known to lower the photosynthetic pigments and protective enzymes in plants (Zhao et al., 2013). Although the pH was low at station 2 during pre-monsoon, it remained within the tolerable limits for plants.

Sediment electrical conductivity (EC) measures the capacity of sediment water to carry electric current and represents the amount of total dissolved solids (TDS). EC also describes the soil salinity (Guang-Ming et al., 2006). Soil EC has an effect on metal bioavailability to plants (Nouri et al., 2009). A significantly high EC values at station 2 and 3, especially during dry seasons (post-monsoon and pre-monsoon) indicates

the influence of industrial outfalls in this region. Heavy metal contamination from sources such as municipal sewage is also found to influence the EC (Kumari et al., 2013). An elevated value of EC in these regions may also indicate salinity ingression as reported in previous studies (Joy and Balakrishnan, 1989; KSTSCE, 2009). High EC values at station 1 during monsoon might be due to the land runoff.

It is not surprising to see that EC and pH are negatively correlated (though not significant at any stations) since a decrease in pH (ie, increased H^+ ions) results in increased conductivity. However, the fact that the presence of heavy metals can also increase the EC should also be noted as shown by Kumari et al. (2013) in a study on river Ganges. The relationship between EC and pH were not strong enough to demonstrate the presence of dissolved solids, especially metals in these regions. The mineralization of waste and organic matter can be a factor influencing the soil EC values (Carmo et al., 2016).

Consistently lower values of ORP at station 3 during the non-monsoon season (when the river flow is low) might probably be due to higher TOC content and the associated anaerobic condition in sediments from this region. This is because the O_2 deficient conditions in sediments where the organic matter is abundant lead to the consumption of compounds other than O_2 by microbes resulting in low redox potential (Gardiner and James, 2012). ORP shows the degree to which a substance oxidises or reduces another substance.

The restricted river flow at Eloor station due to the presence of a check dam at Binanipuram compared with unrestricted river flow at station 1,

especially during non-monsoon, might have resulted in well oxygenated condition and subsequent increase in ORP at station 1. This may also explain the negative correlation between TOC and ORP at station 2 in which the lack of oxygenation and the presence of decaying organic matter might have stimulated the reduced condition. Generally, a negative correlation between ORP and TOC may be explained by the reducing nature of sediment matrix as a result of O_2 consumption in the organic content rich environment (Gardiner and James, 2012). However, several other factors also influence the ORP. For example, changes in ORP and pH in sediment are controlled by factors including chemical inputs, soil vulnerability, and land use policies (Bourg and Loch, 1995). The human interference with the environment can have a major influence on pH and ORP with the subsequent effect on the solubility of heavy metals (Bourg and Loch, 1995). High ORP at station 2 during post-monsoon despite the low TOC, compared to other stations can be suggestive of heavy metal pollution.

According to Stumm and Morgan (1996), sediment and water comprise four characteristic ranges of redox potentials. The first range of 710 to 800 mV (at pH 7 to 8) represents water with sufficient amount of oxygen. The second, low oxygenated range (-100 to 710 at pH 7 to 8) represents the reduction of Fe III, and Mn (III and IV) to Fe II and Mn II, respectively. The range three is characterised by FeS and MnS forms followed by the anoxic condition at range four. The low pH resulting from oxidation of sediment releases metals like Al, Pb, Zn, and Ca into the water (Miao et al., 2006).

In a previous study, an increase in sediment TOC towards the estuarine region of river Periyar during post-monsoon has been reported (Saraladevi et al., 1992). Generally, due to excessive streamflow in the river, a lesser amount of organic carbon content in sediment is observed during monsoon. The present study, however, indicated a slight reduction in TOC content at stations downstream towards the estuary during post-monsoon. The result indicates the influence of anthropogenic inputs of TOC in the region of river surrounding the industrial belt during wet seasons. According to Thottathil et al. (2008) the estuarine regions of Kochi shows a decline in the dissolved organic carbon content during post monsoon. Huge inputs of nutrients in this region from various sources including industries irrespective of seasons, as suggested by Madhu et al. (2007) might be one of the reasons for high organic carbon content downstream of the industrial area. He also noticed that, due to the scarcity of phytoplankton grazers, a major portion of the carbon in the Cochin estuary remained as unconsumed primary production during fresh water dominated seasons. Ideally, sediments with higher grain size are associated with lower organic carbon content (Wakeel et al., 1957). Thus, slightly higher values of sand at station 2 during post-monsoon may explain lower values of TOC content at this station. Highly toxic nature of sediments, due to the close proximity of industries near this station, might have wiped out the benthic vegetation in this region leading to a TOC deficient condition.

Relatively high TOC at station 3 may be due to the heavy load of pesticide containing effluent drained into the Kuzhikandam thodu by the pesticide factory nearby. It has been reported that higher pH and

reducing atmosphere favours pesticide degradation (Pingali and Roger, 1995; Singh, Walker, et al., 2003). The carbon containing intermediates produced by the degrading pesticides under alkaline pH at this station may be contributing to the TOC. The water in the Kuzhikandam thodu remains black in colour throughout the year indicating high carbon content which may further support the presence high TOC at this station. A strong correlation between TOC and pesticides in sediments has been reported by Hung et al. (2007). Toxicity of pesticides, except those like Endrin, reduces as the TOC in the sediment increases (Nebeker et al., 1989).

Though the alkaline pH favours the release of phosphorus (Li et al., 2013) at station 3, the available P fraction may be limited due to its irreversible complexation with the organic matter under certain conditions (Haque et al., 2013). This may partly explain the lack of difference in available P among the stations despite the presence of high organic content and pH at station 3.

Heavy metals are generally more toxic at lower pH and hence it is less likely to cause toxicity under the prevailing high pH at station 3. However, there are instances where high pH can also enhance metal toxicity (Dave, 1985; Michnowicz and Weaks, 1984; Schubauer-Berigan et al., 1993).

Potassium and phosphorus showed an irregular pattern during sampling periods. However, Maya and Maya and Seralathan (2005) observed an increasing trend of K in bulk sediments towards the estuarine regions of the River Periyar. Potassium, an important nutrient element, is derived from potassium feldspar (Larsen and Chilingar, 1979). The leaching,

absorption, fixation, and release of K are governed by the clay content as well as the type of minerals present in it (Mengel et al., 2001).

Phosphorus is an important vital element and represents a component of several macromolecules in the living system. The major sources of P in sediment can be traced to urban and agricultural activities (Carpenter et al., 1998). The increase in temperature, pH, and DO enhances P release from the sediment to the water (Wu et al., 2014).

Ramesh et al. (2015) has reported a more than two-fold increase in the concentration of dissolved inorganic phosphorus in Indian rivers compared with other rivers around the world, which he attributes to the anthropogenic sources like industries. Joseph (1974) observed an increase in total phosphorus in the estuarine region of Periyar and a decrease at the Binanipuram region which is located at the industrial belt. Later studies, however, reported higher total phosphorus concentration in the vicinity of Binanipuram region (Joseph et al., 1984; Joy and Balakrishnan, 1989). These studies have pointed out the industries as the main source of phosphorus. The data on available phosphorus obtained in the present study do not agree with the distribution pattern of total phosphorus in the study area as explained by these authors. In fact, the amount of available fraction of phosphorus is determined by several factors. At present, no sufficient data regarding the available fraction of phosphorus in sediments from the River Periyar is exists and further studies are warranted in this regard.

Sediment texture is one of the factors that define the benthic community associations (Parsons et al., 2013). The heavy metal transport and

storage within fluvial sediments depends mainly on clay and silt fractions (Zhang et al., 2014). The fine particles adsorb and transport metals from water to bottom sediments diagenetically (Zhang et al., 2014). In a previous study, Saraladevi et al. (1992) have reported brownish and blackish nature of sediments from the upstream and the downstream region of effluent discharge point at Binanipuram (S2), respectively, indicating the oxidised condition of sediments. However, in the present study, yellow-brownish sediments were recorded from the point of industrial discharge, which may indicate the presence of contaminants. Moreover, the sediment texture at all the sampling stations, was found to be dominated by sand without any significant variation across stations or seasons. This finding contrasts with the findings of Saraladevi et al. (1992) who reported an increase in clay content towards the bar mouth.

Nevertheless, as observed by Saraladevi et al. (1992), the sand fraction at S2 (Binanipuram region) was slightly higher during post monsoon. The sediments from this region had an unpleasant smell that sediments do not have naturally. This might be due to the industrial discharge of effluents from the factories nearby. Since no significant temporal or spatial changes in sediment textural classes.

The lower values of TS content at station 3 indicates the muddy nature of sediments in this region. A characteristic foul smell of sediment from this region might probably be due to a constant input of effluents containing chemicals from factories such as HIL, FACT, and Merchem.

Significantly high growth in sediments from station 1 indicates that this region is relatively less polluted. The station 2 and station 3 did not

differ in toxicity in most of the cases. However, this may not imply that the same toxicants are responsible for the toxicity at these sites as it is evident from the relationship between sediment chemistry and biological response of *O. sativa*.

The presence of significant interaction effect observed for shoot growth ndicate its sensitivity in detecting minute differences in sediment chemistry with seasonal changes. Such varied toxic response of different organs can be exploited in toxicity characterisation of toxicants. The difference in response pattern between root and shoot may indicate the difference in their sensitivity to the same toxicant. It may also indicate the presence of different toxicants to which root and shoot respond differently. Pronounced toxicity during the dry season in most samples is due to settling down of toxicants in sediments and lack of diluting process like rainfall (Suares-Rocha et al., 2015). This may also explain the slight reduction in toxicity during pre-monsoon when the river received brief rainfall.

The temperature rise during summer also contributes to the toxicity by changing the bioavailability of contaminants as well as the metabolic rates of organisms. A strong negative correlation between EC and biological responses at station 3 suggests the involvement of dissolved solids in toxicity. Though salinity ingression should not be ruled out as a cause of toxicity, especially during post-monsoon, the results from the Vyttila-6 (a salinity tolerant rice variety) make it unlikely to be the main cause. Moreover, in most samples, the toxicity persisted regardless of the season. This may also indicate other sources than the salinity as the cause of toxicity. Additionally, none of the samples exceeded the threshold limit

(3 dS/m) of salinity suggested for *O. sativa* (Linh et al., 2012; Mass and Hoffman, 1977). Moreover, the presence of toxicity in sediments from station 2, which lies away from the influence of salinity (i.e., upstream of the check-dam at Binanipuram), also indicate other sources as toxicants. The lack of significant correlation between EC and growth responses at the station 2 further strengthens this fact. To sum up, it can be argued that the toxicity in the lower reaches of the River Periyar, at least at station 2, is caused by metals or some other unknown toxicants. The results presented here call for further investigations in this regard.

The study also shows that *O. sativa* can be used as an excellent tool for sediment toxicity assessment. In a previous study by Brinke et al. (2015), pre-germinated seedlings of *Oryza sativa* were exposed to sediment from the river Rhine in Germany. However, this method lacks realism as it skips the early stages of seed germination. Unlike the method used by Brinke et al. (2015), the present investigation involved the direct exposure of rice seeds to the contaminated sediment, which is more simplistic, efficient, and cost effective method.

The lack of elutriate toxicity (despite the presence of whole sediment toxicity) at station 3 indicates that the contaminants are strongly associated with the solid phase or pore water of the sediment. This may suggest the possibility of hydrophobic toxicants in the sediment from station 3. Harkey et al. (1994a) has reported that highly hydrophobic contaminants are less soluble in elutriates. Generally, the contaminant bioavailability is high in aqueous phase, especially pore water (Harkey et al., 1994b; Mothersill and Austin, 2003). Nevertheless, several studies

with plants, animals and microbes have reported elutriate to be generally less toxic than whole sediment (Baran and Tarnawski, 2015; Burgess and McKinney, 1997; Van Beelen, 2003). Nalewajko et al. (1989), in his study using algae, marked that phosphates present in the elutriate can bind with the metals and can thus reduce the metal toxicity. He also stressed the possibility of hydrophobic contaminants for low toxicity in elutriates. Pesticides are generally hydrophobic. The presence of pesticide factory in the vicinity of sampling station 3 strongly suggests the possibility of pesticides as one of the causes of toxicity.

4.5 Conclusion

O. sativa was successfully utilized to assess the toxicity of sediments from the River Periyar. The toxicants seem to vary with regard to sampling stations. The study revealed that the sediments at station 2 and further downstream are sufficiently phytotoxic to cause severe damage to plants. This may affect the entire plant community and the associated fauna of this region. Though the sensitivity of different morphological responses of the test species varied to some extent, all of them were efficient in detecting sediment toxicity. Sediment toxicity at station 3 could partly be attributed to dissolved solids. The exact cause of sediment toxicity at station 2 still remains to be elucidated. Morphological responses of *O. sativa* in sediments from station 1 clearly indicate that this region represents a relatively pristine environment. The study also points to the importance of integrating bioassays (which is rarely observed in Indian scenario of risk assessment studies) in sediment assessments rather than

relying only on traditional sediment chemistry-based methods. In short, though the study points towards some possible factors as the cause of toxicity, further investigations (such as toxicity identification evaluation) are necessary to reach a plausible conclusion.

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Chapter 5

Sediment Toxicity Identification Evaluation (TIE) of the River Periyar with *Oryza sativa*

5.1 Introduction

Sediments are the store house of billions of contaminants which poses the risk of being transferred through food web and accumulated at different trophic levels. The management and remediation of sediment is an expensive and daunting task. Sediment quality guidelines (discussed in chapter 4) form the basis for sediment management and cleaning up activities.

However, the present SQGs provide only generalized information, and are not sufficient to address the exact cause of toxicity. The knowledge about the exact cause of toxicity will save time, effort, and cost required in prioritizing the sediment management and cleanup activities (USEPA, 2007). Sediment toxicity tests, which only quantify the toxicity, do not provide sufficient information about the contaminants responsible for toxicity. This is because it is difficult to relate the toxicity to a particular compound among the innumerable numbers of co-occurring chemicals present in the contaminated sediment (Ankley and Schubauer-Berigan, 1995). To overcome this issue, USEPA (1991, 2007) has developed a toxicity identification evaluation protocol (TIE) in which the sample is purposefully manipulated and subsequently bioassayed to specifically identify the compound causing toxicity (see chapter 3). The advantages of sediment TIE are many fold. The results obtained from TIE can be used by regulatory authorities in the development of permit limits for discharge, safe disposal of dredged material, and improving sediment quality guidelines (Ankley and Schubauer-Berigan, 1995).

Like effluent TIE, sediment TIE also has three phases (Fig. 5.1): characterisation (Phase I), identification (Phase II), and confirmation (Phase III). The sediment TIE can be performed using whole sediment, pore water or elutriate, though each method has its own merits and demerits. The whole sediment TIE method is somewhat different from those performed with aqueous fraction (pore water and elutriate), though there may be a convergence at later phases.

Sediment TIE starts with an initial toxicity test to check for the pres-

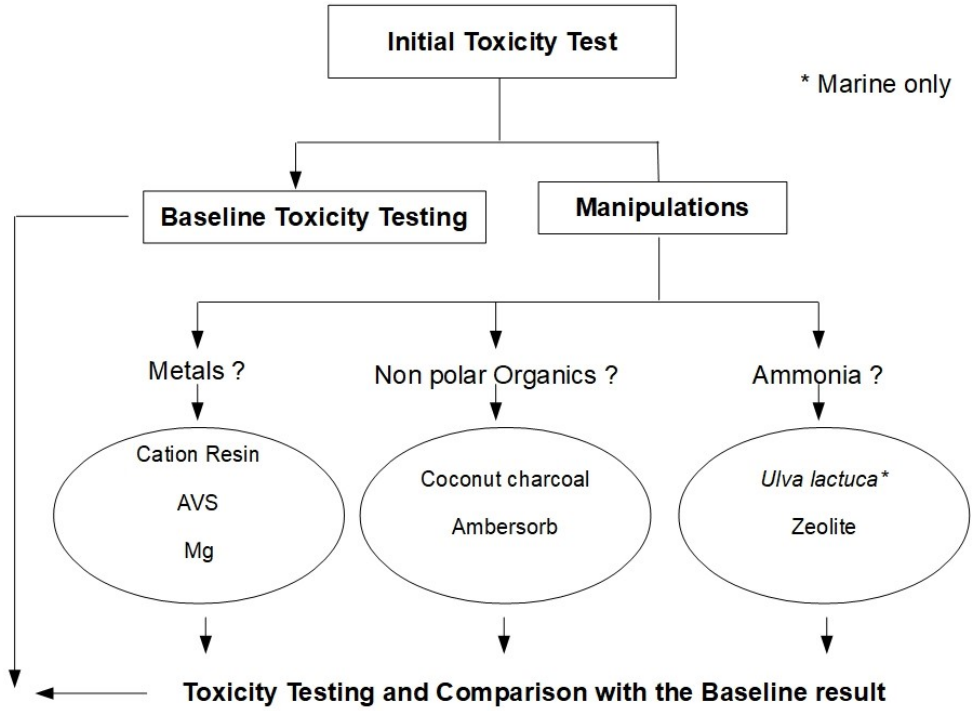


Figure 5.1: TIE approach with whole sediments.

ence of toxicity, and to decide the endpoints and the duration of exposure to be chosen. The next step is to begin Phase I TIE manipulations along with a concurrent baseline test which represents the untreated, contaminated sediment. The baseline test is required to evaluate the change in toxicity by comparing it with TIE manipulations. The Phase I TIE manipulations involve the following sediment treatments targeted at ammonia, cationic metals, and non-ionic organics, which are frequently found in sediments:

a) Zeolite addition (or *Ulva lactuca* addition for marine TIEs) to remove ammonia.

b) cation exchange resin (CER) addition, and/or acid volatile sulfide (AVS) addition to remove cationic metals.

c) Coconut charcoal addition and/or ambersorb (a type of resin) addition to remove non-ionic organics.

To date, most TIE studies have been performed with animals including cladocerans and fishes, echinoderms, bivalves, and gastropods (Ankley and Schubauer-Berigan, 1995; Hogan et al., 2005). Although, TIE methods have recently been developed for some algal species (Hogan et al., 2005; Kim et al., 2015; Strom et al., 2009), they are more suitable for TIE with aqueous extracts than solid phase sediments.

While there have been some efforts to include vascular plants in toxicity tests, the use of vascular plants in TIE remains neglected. Some efforts have been made recently to include terrestrial vascular plants like *Lycopersicum esculentum*, *Lactuca sativa*, and, *Raphanus sativus* in TIE of land fill leachate, swine slurry and spiked water (Budi et al., 2016; Fjällborg et al., 2006; Villamar et al., 2014). However, the utility of these plants in whole sediment TIE has never been explored. Moreover, these plants require additional supporting substratum (e.g., filter paper) to survive on whole sediment as they are terrestrial species for which sediment is not a suitable substratum normal growth. An alternative is to use aqueous extracts (elutriate and pore water) of sediments for exposure, but it has been shown that the aqueous fractions of sediments

are prone to several artefacts which may affect the results (Ankley and Schubauer-Berigan, 1995; Burgess, Ho, et al., 2011; Chapman et al., 2002).

Oryza sativa is best suited for sediment toxicity tests as it grows well in swampy areas. Besides several benefits of using macrophytes like rice in sediment toxicity tests, there is an added advantage that it requires no additional supporting substratum for growth in sediment and therefore, gives more reliable results than those obtained from seed germination tests using filter paper. Seed germination tests requires only small volume of sediment (usually <= 20 g/wt weight) when compared with the USEPA recommended, scaled down versions of TIEs using other organisms (USEPA, 2007).

In North America, recent developments in environmental regulations includes the identification of environmental stressors, which encourages the use of methods like TIE (Burgess, Ho, et al., 2011). Asian countries including India still rely on chemistry based methods for sediment quality assessment. At present no published reports on sediment TIE is available from India.

The River Periyar is perhaps the only river in Kerala that receives the highest amount of effluent load. Hundreds of gallons of effluents are released daily from Eloor – Edayar industrial region located in the lower reaches of this river. The River Periyar, especially at the Eloor – Edayar region, has been shown to be contaminated with metals, pesticides, and radionuclides (Anu et al., 2014; Balachandran et al., 2003; Balakrishnan et al., 2016; Charuvilayil, 2013; DineshKumar, 1997; George et al., 2016;

Kumar et al., 2011). However, these reports are based on sediment/water chemistry which cannot reveal the exact cause of toxicity to organisms. Therefore, it is necessary to include methods like TIE in sediment quality assessment to provide a clear understanding of the nature of contaminants responsible for toxicity.

Considering the issues stated above, the present study attempts to standardise and validate sediment toxicity identification evaluation using *Oryza sativa*. This is also the first report on sediment TIE of the River Periyar.

5.2 Materials and Methods

The whole sediment TIEs were performed in two steps, the first step being the standardization of Phase I manipulations for the test species, and the second step being the validation of the protocol using contaminated sediment from the River Periyar (identified in the sediment toxicity assessment study of chapter 4).

5.2.1 Tolerance of *O. sativa* to Phase I Manipulations of Whole Sediment TIE

Control Sediment

The control sediment is used to assess the quality and performance of the test organisms in the absence of contaminants (USEPA, 2007). Control sediment prepared as per the OECD guidelines (OECD, 2014) contained kaolin clay (Nice Chemicals), quartz sand (purchased from a local aquarium supplier), and alpha cellulose (Sigma Aldrich) at 20, 75,

and 5% (dry weight) respectively. The alpha cellulose functions as an alternate source of organic carbon (USEPA, 2000). Nutrient solution (Smart and Barco medium) was added to this mixture to bring the moisture content of final mixture to 50%. The pH was adjusted to 7 by adding $CaCO_3$.

TIE Manipulations in OECD sediment

To determine the tolerable range of *O. sativa* var. Jyothi to each TIE manipulation, a series of bioassays were conducted with another set of OECD sediment spiked (wet weight basis) with different concentrations of substances used for TIE manipulations. For this, zeolite grain, cation exchange resin (CER), powdered coconut charcoal, and sodium sulfide were used. Zeolite (Nice chemicals) was rinsed well in distilled water before use. Cation exchange resin (axion c220 NA;.3–1.2 mm mesh size) was prepared by rinsing the resin in distilled water ($\backsimeq$ 1:4 v/v), followed by decanting the water. The resin was then re-suspended in 30% NaCl (four volumes) and stored at 4°C for 24 hours. The resin was thoroughly rinsed in distilled water (four volumes) several times before use. A 0.5 ml of Na_2S (sodium sulfide; Merch) solution prepared in distilled water (at different dilutions) was added to each cup to achieve the desired concentration ranges. Powdered coconut charcoal (PCC; $<$ 45 µm) was prepared by mixing distilled water and PCC (40 and 60%, respectively on weight basis) which is subsequently kept under vacuum (4°C) overnight before use.

Bioassays involved either one of the following treatments in OECD

sediment (20 g wet weight/cup): zeolite grain or CER at 20, 10, 5, 1.25, and 0%, PCC at 5, 2, 1,0%, sodium sulfide at 20, 10, 5, and 0% of wet weight sediment. The sediment was equilibrated for 24 hours before the seeds were added.

5.2.2 Whole Sediment TIE

Sediment Sample

The sediment sample was collected from the sampling station 2 (S2; Binanipuram, Eloor) which was observed to be toxic in the sediment toxicity tests (see chapter 4). This station was chosen because the influence of salinity ingression was less in this region when compared with station 3 (S3) which lies closer to the bar mouth. Sampling was done on May 2014 with a Van Veen grab.

TIE Manipulations in Contaminated Sediment

Sediment manipulations consisted of spiking the contaminated sediment (S2) with zeolite grain (20%), CER (20%), PCC (5%), and sodium sulfide (20%). A dilution blank (consisting of contaminated sediment spiked with 20% quartz sand) was also included to check whether the toxicity reduction is due to the dilution of sediments by the added substances. Toxicity tests began after 24 hours of equilibration. A baseline test (untreated toxic sediment) was run concurrently to compare the effect of TIE treatments on toxicity. The sediment TIE was repeated with dried samples to evaluate the presence of volatile compounds. The sediment was shade dried for 5 days and were sieved through a 0.64 mm sieve. The

treatment involved baseline, CER addition and dilution blank only; this time both the dilution blank and CER were reduced to 10% due to their intrinsic toxicity.

All experiments (both standardisation and validation) were performed in polypropylene plastic cups (250 ml; 90 × 5 mm). The standardization experiments consisted of 3 replicates whereas validation experiments consisted of 5 replicates. Each test vessel contained 20 g of sediment and 10 seeds. Bioassays began only after 24-hrs of equilibration period. Both standardisation and validation tests were run at 28±2°C with a 14-hour light and 10-hour dark cycle (300 µmol/m^2/s) for a duration of 96 hours. All measurements were made using Fiji software (Schindelin et al., 2012).

Metal Analysis

Dried, sieved sediment samples were used for available metal (Fe, Cu, Mn, Zn, Mg, Cd, and Ni) analyses. DTPA extraction was used for available Cd, Zn, Fe, Mn and Cu, Ni (Lindsay and Norvell, 1978; Staff, 1996), whereas ammonium acetate extraction was used for available Mg (Staff, 1996). The metals which demonstrated good correlation with toxicity was further analysed for total concentration. For total metal analyses, sediments (at the end of bioassay) from the test vessels containing CER treatment were sieved again through a 0.64 mm sieve to separate the CER retained on the sieve. The resin was eluted as per the methods suggested by USEPA (2000). Metals were analysed using AAS (atomic adsorption spectroscopy).

5.2.3 Data Analysis

One-way ANOVA followed by Dunnet's multiple comparisons (multcomp package in R) were used (Hothorn et al., 2008) in standardization tests to compare the control sediments with each concentration of TIE treatments. One-way ANOVA followed by Tukey's HSD using lsmeans package (Lenth, 2016) was used to analyse results from the validation study. Since seedling and shoot data did not meet the normality assumptions, they were transformed using natural and common logarithms, respectively. In cases where parametric assumptions were violated even after transformation (e.g. root in river sediment), Kruskal-Wallis test followed by Dunn test were performed using FSA package in R (Ogle, 2017). Both available and total concentrations of metals were correlated with the growth responses of plant to see if there is any relationship between metal content in the sediment and plant growth.

5.3 Results

5.3.1 Tolerance of *O. sativa* to Phase I Manipulations of Whole Sediment TIE

The root, shoot and seedling lengths in control sediment at day 4 were 57±12.42, 29.29±5.21, and 87.20±1.42 mm respectively. In general, shoot was least affected by various TIE treatments.

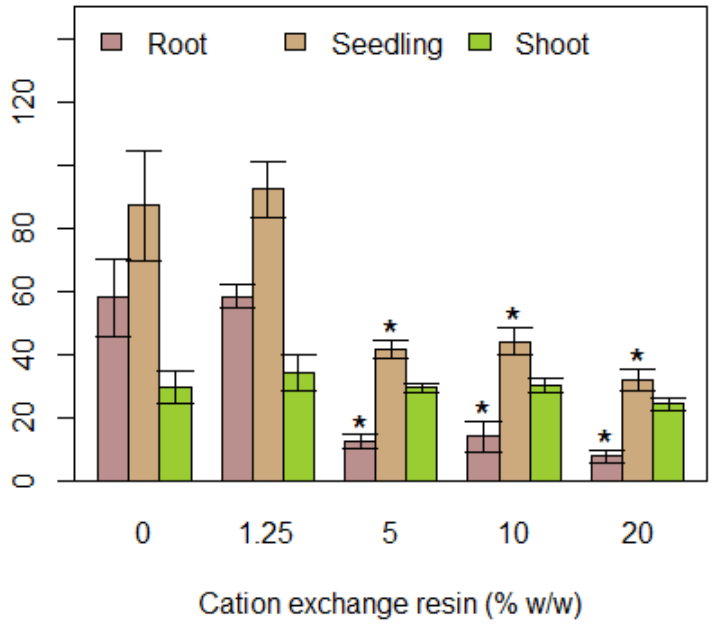


Figure 5.2: Mean length of root, shoot, and seedling lengths of *O. sativa* exposed to cation exchange resin in OECD sediment. Asterisks denote significantly lower values relative to control ($p < 0.05$; ANOVA followed by Dunnet's multiple comparison test). Error bars represents standard deviation.

Tolerance of *O. sativa* to Cation Exchange Resin (CER) Treatment

Root tolerated the cation exchange resin concentration up to 1.25%, beyond which a sharp decrease in root length was observed ($p < 0.001$).

However, none of the resin concentrations affected the shoot growth ($p > 0.05$). The shoot length was 24.29 mm in 20% resin (Fig. 5.2).

Seedling produced a growth pattern similar to that of the root. The seedling growth inhibition was significantly increased to 63.52% at 20% resin treatment. In the further TIE using natural sediment, a 20% (wet weight) resin was used to detect heavy metal toxicity.

Tolerance of *O. sativa* to Zeolite Treatment

The response of all the growth parameters in zeolite treatment resembled that of cation exchange resin treatment. Both root and seedling demonstrated a significant growth reduction at 5% zeolite and onwards ($p < 0.001$). Root demonstrated the highest inhibitory effect (86.52%) at the top concentration (Fig. 5.3). Zeolite caused no significant adverse effect on shoot growth. Twenty percent zeolite was used in further sediment TIE.

Tolerance of *O. sativa* to Sulfide Treatment

The results showed that the sodium sulfide treatment up to 10% did not affect any of the growth parameters studied (Fig. 5.4). A statistically significant decrease ($p < 0.001$) in growth for all the parameters was observed at 10% followed by further inhibition at 20%. For all the parameters, the inhibitory effect of sulfide approached approximately 100% at highest concentration. Despite the blank toxicity observed at 20% sulfide addition, the same concentration was used in TIE with natural sediment. This maintains comparability with previous studies using other organisms.

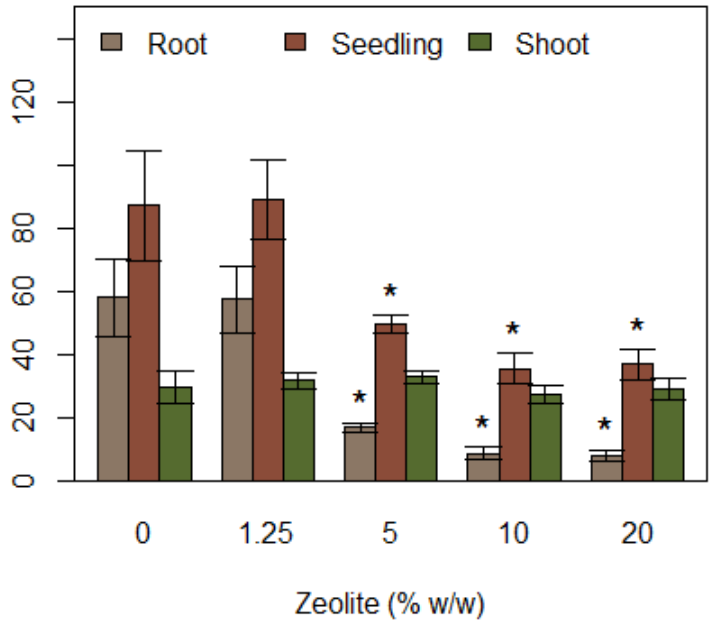


Figure 5.3: Mean length of root, shoot, and seedling lengths of *O. sativa* exposed to zeolite in OECD sediment. Asterisks denote significantly lower values relative to control ($p < 0.05$; ANOVA followed by Dunnet's multiple comparison test). Error bars represents standard deviation.

Tolerance of *O. sativa* to Powdered Coconut Charcoal (PCC) Treatment

Unlike other treatments, charcoal stimulated the growth of root and seedling at all the concentration ranges studied (Fig. 5.5). The growth induction reached up to 71.8% in the case of root ($p < 0.01$). However, the shoot remained unaffected even at higher concentrations. The growth stimulation occurred at the concentration ranges recommended for fine-

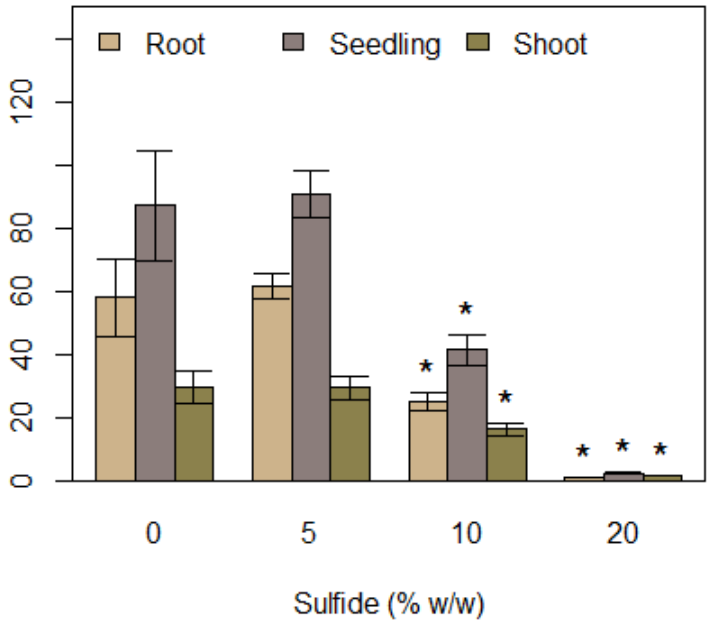


Figure 5.4: Mean length of root, shoot, and seedling lengths of *O. sativa* exposed to Na_2S in OECD sediment. Asterisks denote significantly lower values relative to control ($p < 0.05$; ANOVA followed by Dunnet's multiple comparison test). Error bars represents standard deviation.

grained charcoal by USEPA. The problem of growth induction in the blank can be dealt with by including a charcoal blank and using a blank correction method in the sediment TIE.

5.3.2 Whole Sediment TIE of the River Periyar

The Kurskal Wallis test revealed significant effect of TIE treatments on root length (chi-squared = 28.305, df = 6, $p < 0.001$). The post hoc test (Dunn test) showed that adding sulfide and charcoal significantly reduced

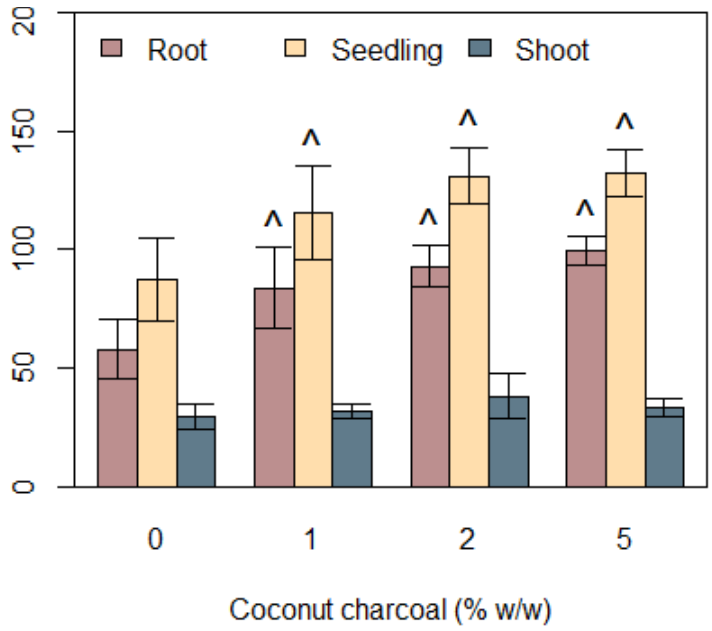


Figure 5.5: Mean length of root, shoot, and seedling lengths of *O. sativa* exposed to coconut charcoal in OECD sediment. Arrow heads denote significantly higher values relative to control ($p < 0.05$; ANOVA followed by Dunnet's multiple comparison test). Error bars represents standard deviation.

the sediment toxicity (Z = -3.27, $p < 0.01$; Z = -2.62, $p < 0.05$). The root length in baseline was only 13% of control (Fig. 5.9). CER and zeolite caused no significant reduction in toxicity. However, a slight increase in root length, compared with baseline, was observed (mean = 32.7 and 35.5% of control for CER and zeolite, respectively).

Sulfide addition resulted in the highest reduction in toxicity (Fig. 5.6) as indicated by the increase in root length (71.3% of control). The length

in dilution blank was not statistically significant from the baseline for any of the response variables studied ($p > 0.05$).

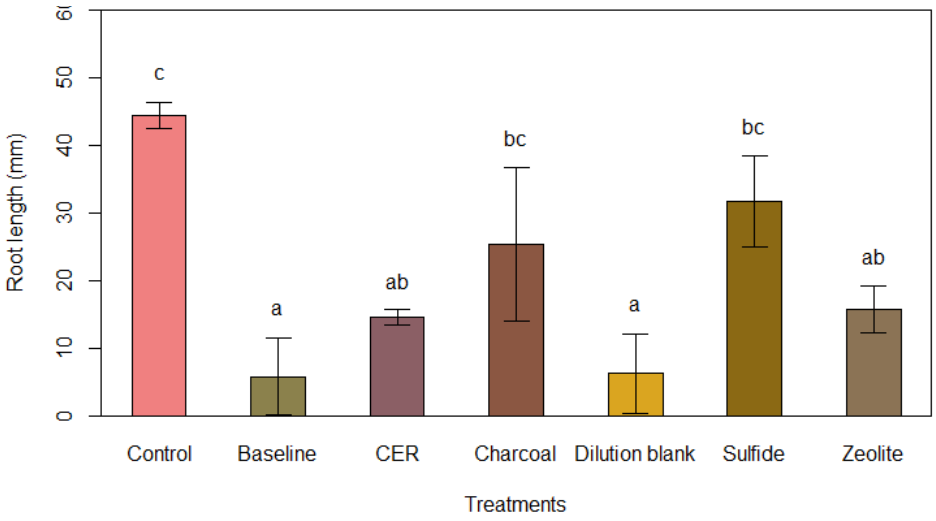


Figure 5.6: Mean root length of *O. sativa* in sediment TIE manipulations. CER = cation exchange resin. Dilution blank contains river sediment diluted with 20% quartz sand. Values followed by the same letter do not differ significantly ($p > 0.05$, Kruskal-Wallis followed by Dunn test). Error bars represents standard deviations.

In the case of shoot growth also, none of the treatment except charcoal and sulfide caused a significant improvement (back-transformed mean = 68.9 and 57.9% of control, respectively; Fig. 5.7). Though the shoot growth in dilution blank was slightly greater than that of the baseline, it was statistically non-significant ($p > 0.05$). The seedling too produced a response pattern similar to that of root with a significant growth improvement in charcoal and sulfide addition tests (Fig. 5.8).

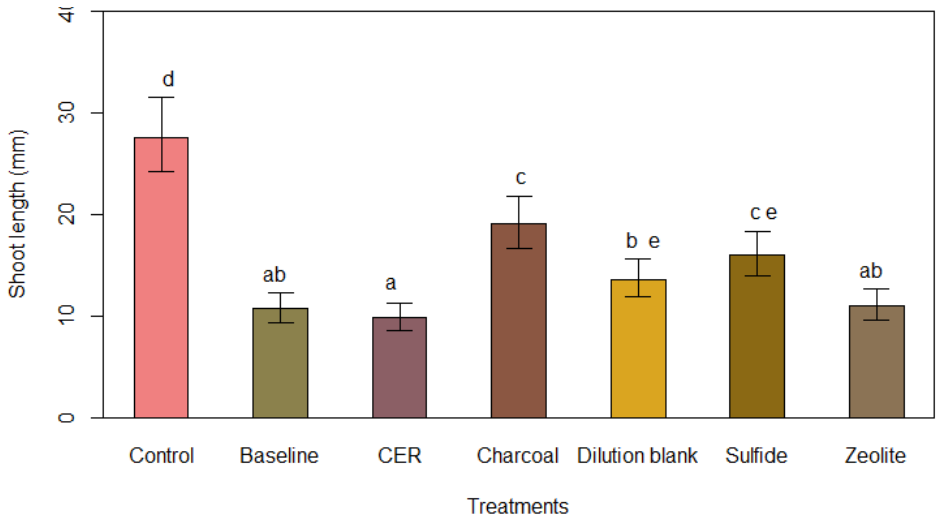


Figure 5.7: Mean (back-transformed) shoot length of *O. sativa* in sediment TIE manipulations. CER = cation exchange resin. Dilution blank contains river sediment diluted with 20% quartz sand. Values followed by the same letter do not differ significantly ($p > 0.05$, ANOVA followed by Tukey's test). Error bars represents standard deviation.

Drying the sediment led to significant ($p < 0.01$) increase in root length in CER when compared with its baseline (Fig. 5.10). The root length in dilution blank did not differ from baseline significantly. As with the wet sediment TIE, CER addition in dried sediment did not produce any significant difference when compared with shoot length of baseline; shoot, however, showed a slight increase in length in CER treatment. Similar to the root length, the seedling length was significantly increased by adding CER.

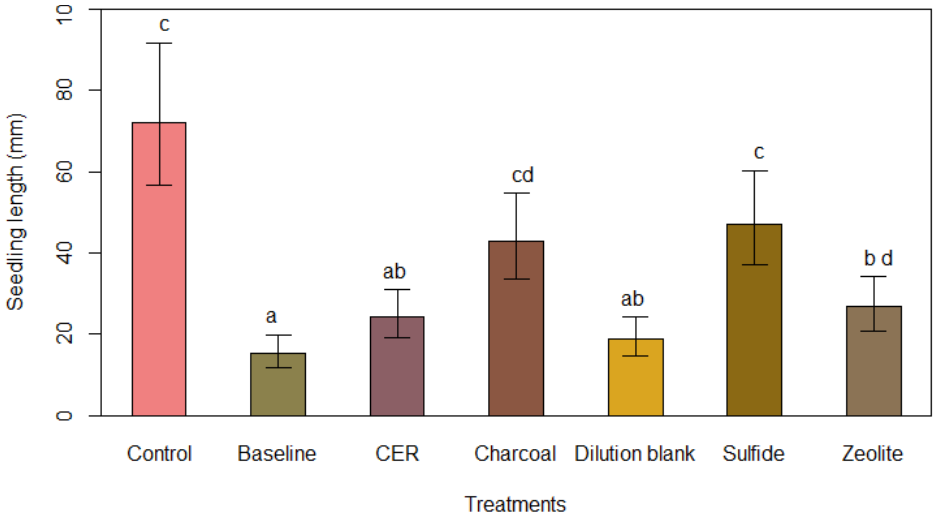


Figure 5.8: Mean (back-transformed) seedling length of *O. sativa* in sediment TIE manipulations. CER = cation exchange resin. Dilution blank contains river sediment diluted with 20% quartz sand. Values followed by the same letter do not differ significantly ($p > 0.05$, ANOVA followed by Tukey's test). Error bars represents standard deviation

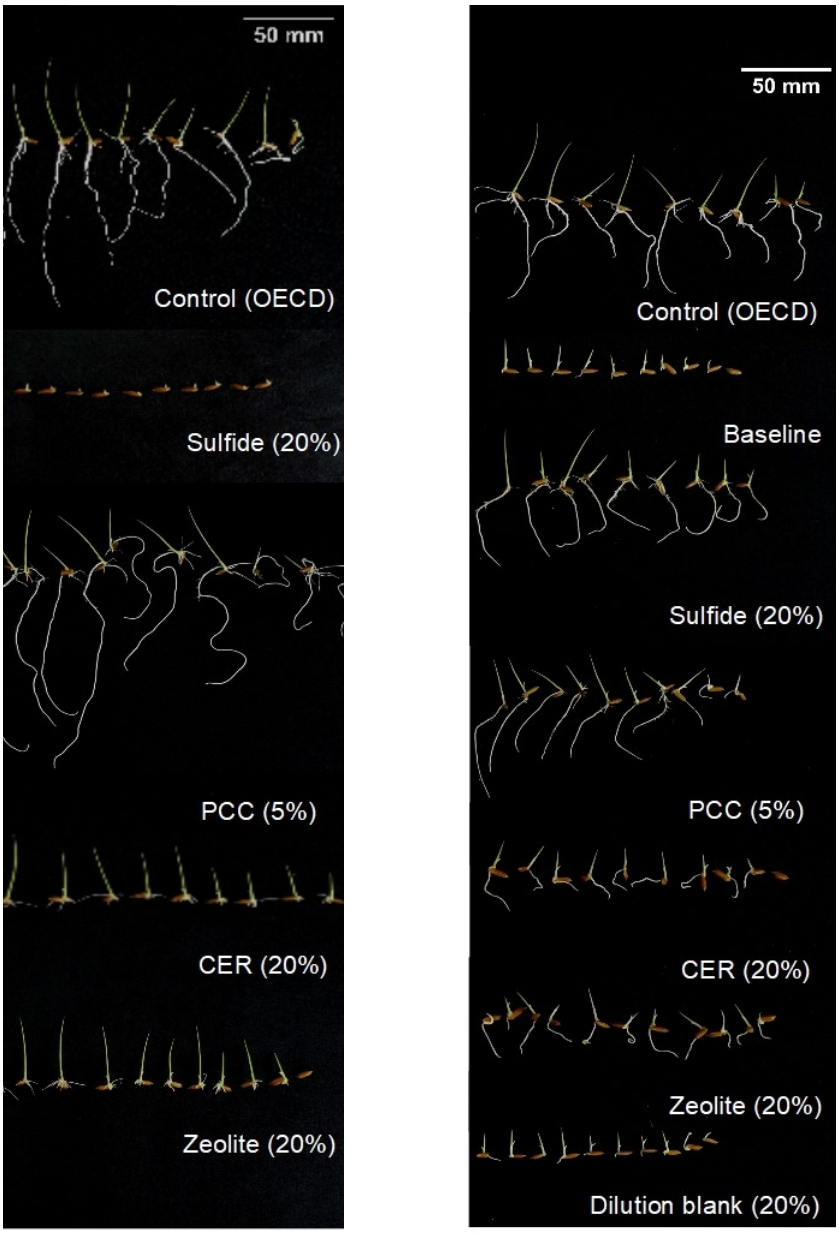
Metal analyses (available as well as total) were performed to investigate the relationship of metals with sediment toxicity. Except for Pb, the available concentrations of all the metals analysed were above the detection limits (Table 5.1). Among metals, Cd and Mn (r = -0.9, $p < 0.05$) showed a strong negative correlation (Table 5.2; Fig. 5.11). The correlation analysis showed that root and seedling lengths were negatively correlated with available Cd (Table 5.3) concentration in the sediment ($r \backsimeq$ -0.9 with $p < 0.05$ for both root and seedling). Moreover, the available Mn

showed a positive correlation with root length and seedling length ($r \backsimeq 0.9$ with $p < 0.05$ for both root and seedling). Though shoot length demonstrated a strong relationship with Cd and Mn, it was statistically not significant ($p > 0.05$). For all the growth parameters, Zn yielded negative correlations whereas Mg and Ni yielded positive correlations; but these relationships were statistically non-significant.

Among the available metals analysed, only Cd was found to show significant correlation with toxicity; hence, only Cd was analysed for total concentration. The total Cd concentrations in replicate samples ranged from 3.6 to 6.8 mg/kg with an average value of 4.52 mg/kg. The total Cd concentration was several folds greater than the recently published consensus-based SQG limit (0.99 mg/kg) and closer to the ERL (effects range-low) limit (5 mg/L) (MacDonald et al., 2000). Further statistical details are available in Appendices D1 to D4 .

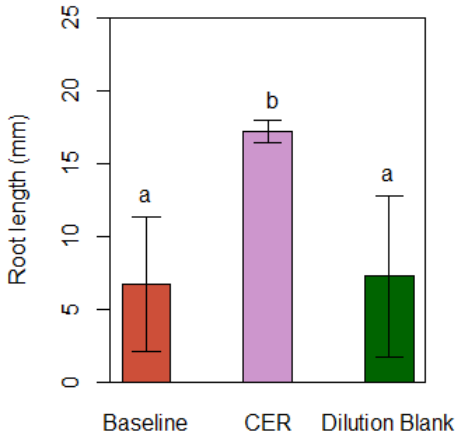
Table 5.1: Available metal concentrations (mean ± standard deviations in mg/kg) in sediment from station 2 (S2, Binanipuram) of the River Periyar.

Fe	Cu	Mn	Zn	Mg	Ni	Cd
8.4	2.68	77.4	101.82	152.07	2	0.1
±	±	±	±	±	±	±
1.6	2.9	60.4	2.2	63.2	0.8	0.1

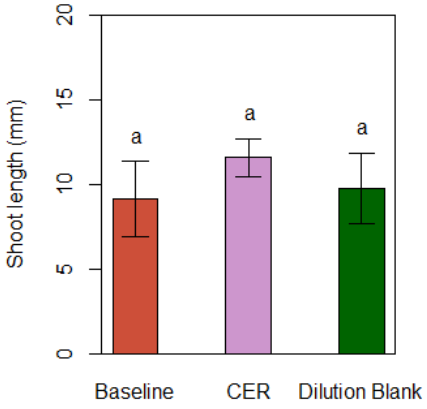


(a) OECD sediment **(b)** Contaminated sediment

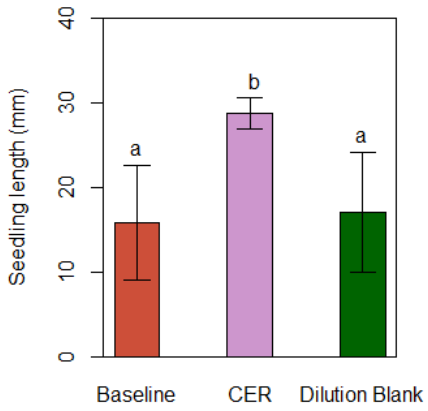
Figure 5.9: Rice seedlings exposed (96-h) to OECD sediments (a) and contaminated (b) sediments (S2; Binanipuram, Periyar) both of which had undergone TIE manipulations.



(a) Potassium.



(b) Potassium.

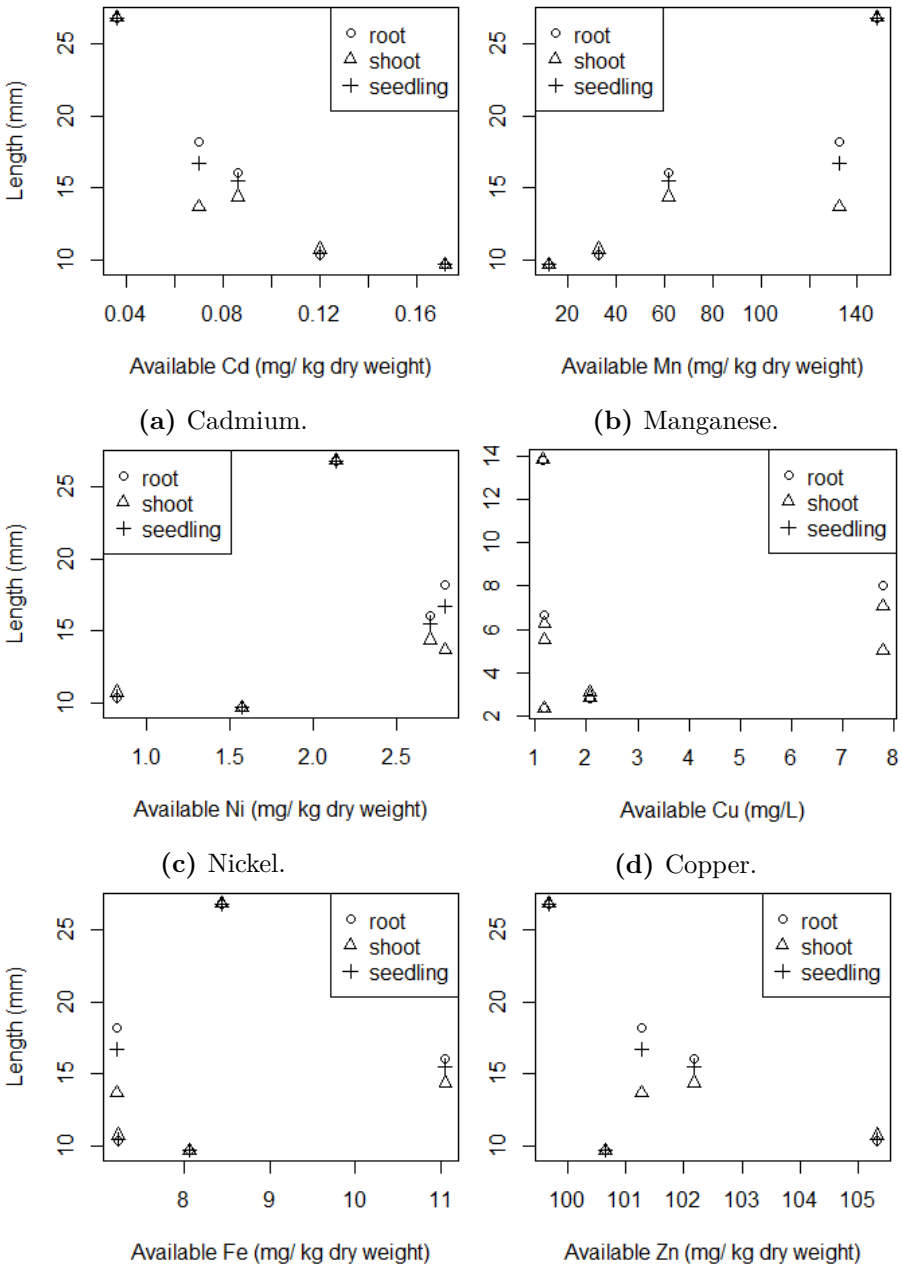


(c) Potassium.

Figure 5.10: Mean root length (a), shoot length (b), and seedling length (c) of *O. sativa* in sediment TIE manipulations with dried sediment. CER = cation exchange resin. Dilution blank contains river sediment diluted with 10% quartz sand. Values followed by the same letter do not differ significantly ($p > 0.05$, ANOVA followed by Tukey's test). Error bars represents standard deviation.

Table 5.2: Correlation between available metal concentrations in sediment from station 2 (S2, Binanipuram) of the River Periyar. Asterisk indicate significant ($p < 0.05$) values.

	Fe	**Cu**	**Mn**	**Zn**	**Mg**	**Ni**
Cu	-0.49					
	0.4047					
Mn	-0.08	0.46				
	0.8987	**0.433**				
Zn	-0.16	-0.02	-0.51			
	0.7938	**0.9791**	**0.378**			
Mg	-0.21	0.85	0.84	-0.27		
	0.7305	**0.068**	**0.074**	**0.661**		
Ni	0.47	0.44	0.62	-0.58	0.69	
	0.4231	**0.4603**	**0.268**	**0.308**	**0.2**	
Cd	-0.16	-0.26	-0.93	0.3	-0.72	-0.57
	0.7985	**0.6726**	**0.0243***	**0.624**	**0.17**	**0.32**



(a) Cadmium. **(b)** Manganese.

(c) Nickel. **(d)** Copper.

(e) Iron. **(f)** Zinc.

Figure 5.11: The correlation between available metal contents in the sediment (station 2) and morphological responses.

Table 5.3: Correlation between morphological responses and available metal concentrations in sediment from station 2 (S2, Binanipuram) of the River Periyar. Asterisks indicate significant ($p < 0.05$) values.

Variables	Fe	Cu	Mn	Zn	Mg	Ni	Cd
Root length	0.16	0.09	0.92	-0.61	0.59	0.55	-0.92
	0.803	**0.880**	**0.025***	**0.272**	**0.3**	**0.33**	**0.028***
Shoot length	0.17	-0.17	0.79	-0.56	0.34	0.33	-0.83
	0.790	**0.787**	**0.110**	**0.321**	**0.576**	**0.591**	**0.084**
Seedling length	0.16	0.01	0.89	-0.6	0.51	0.48	-0.9
	0.796	**0.988**	**0.043***	**0.282**	**0.38**	**0.409**	**0.039***

5.4 Discussion

The toxicity in CER treatment in OECD sediment indicates that the resin might have rendered micronutrients present in the control sediment unavailable to the plant leading to growth inhibition. Zeolite caused no significant adverse effect on shoot growth and this feature can be effectively exploited in TIE of ammonia in natural sediments. For charcoal, the growth stimulation occurred at the concentration ranges generally recommended by USEPA. This provides an advantage of using higher quantities of charcoal that produces a response closer to that of the control sediment. The blank toxicity (in OECD sediment) of TIE manipulations to root does not prohibit the use of root elongation test in TIE, but

rather in combination with shoot data, it becomes a valuable tool in identifying compounds that elicit differential responses in shoot and root. Moreover, blank toxicity is acceptable in TIE studies especially when the corresponding TIE manipulations of toxic sediment shows a reduction in toxicity (USEPA, 2007). For CER and zeolite a concentration of 20% was used for further TIE as none of the concentrations of these compounds produced any toxic effect on shoots of *O. sativa*.

A significant growth improvement of plant organs in sulfide and charcoal amended river sediments clearly indicate the involvement of metals and nonpolar organics in toxicity, respectively. Addition of charcoal renders the organic contaminants less bioavailable and thus reduces toxicity (Ho et al., 2004; Phillips et al., 2006). Since charcoal blanks in OECD sediment tests did not produce any hormetic effect on shoot, the hormetic artefact as the cause of reduction of toxicity (in the case of shoot) in charcoal amendments (in river sediment) could be ruled out. Since no toxic artefact (unlike the case of root) due to CER was observed in shoot, a reduction in toxicity to shoot may signify the involvement of metals. Even though no significant reduction in toxicity was observed in the CER amended river sediments (wet), a notable increase in root and seedling lengths indicated the removal of toxic metals by these treatments. Lack of significant reduction toxicity in CER amendments made with wet sediments may be due to the presence of some substances which interfere with the adsorption process of CER. It is also possible that the added CER is somewhat toxic to the plant; the results of CER treatments with OECD sediment strengthens this fact. A slight reduction of toxicity in zeolite

treated sediments suggests the presence of ammonia, but to a lesser extent. This may also partly explain the lack of significant reduction of toxicity in CER treatments as ammonia interferes with the CER non-specifically (Burgess, Cantwell, et al., 2000).

In previous TIE studies with animals such as *Chironomus dilutus* and *Hyalella azteca*, it has been shown that charcoal at higher quantity can cause toxicity to animals (Phillips et al., 2006; USEPA, 2007). This limits the usefulness of charcoal in characterising organics when present at very high concentrations since saturation effect can limit the sorption of toxicants by charcoal (USEPA, 2007). The lack of blank toxicity in charcoal amendments with *O. sativa* implies that in TIEs with *O. sativa*, charcoal can be used at greater concentrations than those recommended by EPA for many organisms; thus, problems due to the limitations of sorptive capacity of charcoal can be overcome.

A significant reduction of toxicity in the river sediment (wet) treated with sulfide suggests the presence of metals. This is in contrast to sulfide blanks (in OECD sediment) which was significantly toxic to the plant. The toxicity in sulfide blanks may be explained by the lack of sufficient free ions (e.g. metals) in the OECD sediment to reduce the toxicity of added sulfide by binding to it (USEPA, 2007).

Until recently, compounds such as H_2S have been considered phytotoxins. Recent studies conducted with plants, including rice, have demonstrated the ability of sulfides to alleviate metal toxicity (Ali et al., 2014; Dawood et al., 2012; Mostofa et al., 2015; Wang et al., 2010; Zhang, Tan, et al., 2010). Compounds such as NaHS and Na_2S function as

H_2S donors in reactions involving metals and sulfides form metal sulfides which scavenge the toxic metals. Besides scavenging the heavy metals in sediment, sulfide containing compounds such as NaHS can alleviate the metal toxicity by up-regulating the antioxidant enzymes, which is mediated through H_2S (Mostofa et al., 2015).

Since the CER amendment in dried sediment significantly reduced the toxicity (as indicated by an increase in root length), the involvement of metals as the toxicants could strongly be suspected. Drying the sediment resulted in volatile loss of ammonia or some other compounds which might have interfered with the metal adsorption of CER in wet sediment. Since no significant reduction in toxicity was observed in any of the dilution blanks, the loss of toxicity due to the dilution effect of amendments could be overruled. In short, the results from the sediment amendments (sulfide and CER addition, and charcoal addition) strongly implicated metals and organics as the toxicants. To investigate further, the available and total metal content in the dried sediment was analysed.

The total Cd content was found to exceed the limit values of ERL (effects range-low) and consensus-based sediment quality guideline suggested in published guidelines (Long et al., 1995; MacDonald et al., 2000). However, the sediment is unlikely to cause toxicity to rice at this concentration of Cd unless there exists an interaction between the Cd and other sediment contaminants (Brinke et al., 2015). Nevertheless, the growth parameters demonstrated a strong negative correlation with the available Cd content, which suggests the involvement of synergistic interaction of Cd with some other compounds. It could be assumed that

Cd is synergistically interacting with some other metals (not considered in the present study) or organics present in the sediment. Though the instances of Cd phytotoxicity are relatively rare (Brinke et al., 2015), Cd is known to interact synergistically or antagonistically with other compounds (Luan et al., 2008). Metal such as Al is reported to interact with Cd synergistically (Shamsi et al., 2007). However, most studies have shown that the interaction of Cd with other metals such as Zn, As, Fe and Mn results in antagonistic effect on rice (Hassan et al., 2005; Sebastian and Prasad, 2015; Sun et al., 2008). Only a few reports exist with regard to the combined effect of organic compounds and metals to plants. Organic compounds such as pyrene (Zhang, Dang, et al., 2009), LAS (Singh et al., 1994) and fluoranthene (Li et al., 2013) are known to be synergistic to plants.

5.5 Conclusion

Seed germination tests with *O. sativa* has effectively been employed in sediment TIE. Though some of the TIE manipulations with control sediments caused intrinsic toxicity at higher concentrations, results of TIE treatments with natural sediment was found to be promising. The present study successfully characterised the toxicant into different classes, a process that is costly and difficult to achieve in traditional chemistry-based methods. The presence of hundreds of industries in a short span of area at Eloor–Edayar region also makes it difficult to chemically characterise the contaminants using traditional methods. Sediment TIE revealed that at least Cd or some other metals in combination with some organic compound

is causing toxicity. Also, the concentration of total Cd concentration was found to exceed the established sediment quality guidelines (SQGs).

It is important to note that a 96-h exposure was used in the study. TIE with extended exposure periods is also worth investigating to check the influence of exposure duration on toxicity. This study is only a first step towards TIE with *O. sativa*. Such studies could be extended with other species from different plant taxa to make the results extrapolatable to the whole plant kingdom. A detailed investigation involving the use of latest trends in sediment TIE is required to exploit this species as a candidate for sediment TIEs. The study also paves way for future research that concentrates on toxicity based sediment quality assessments, which have hitherto not been well explored with regard to the River Periyar.

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Chapter 6

General Summary and Conclusion

Wide varieties of plants and animal species are being used as model organisms in ecotoxicology. Agencies like OECD, USEPA, and ASTM provide a list of test species for use in standard toxicity tests. *Oryza sativa* (rice), the staple food grain around the world, has been one of the vascular plants recommended by OECD. However, this plant is not widely explored in ecotoxicological studies. The longer shelf life, simplicity in bioassay, and the ability to grow in a wide range of habitat qualify *O. sativa* as an excellent model species in toxicity tests.

Toxicity Identification Evaluation (TIE) is a protocol recently developed by EPA to specifically identify the toxicants in complex samples by conducting toxicity tests before and after manipulating it physically and chemically. This process narrows down a wide spectrum of toxicants to be screened for into small classes. This reduces the cost, time, and effort required to screen all the chemicals present in the sample. However, the potential of vascular plants to be used as a test species in TIE has not

been explored well. In light of issues mentioned above, the present study focusses on the utility of *Oryza sativa* in ecotoxicological monitoring and toxicity identification evaluation (TIE).

Chapter 1 reviews the use of vascular plants in toxicity tests with special reference to *O. sativa*. In addition to pointing to the problem of using animals as surrogates for plants, this chapter also stresses the relevance of using plants, especially rooted macrophytes in toxicity testing. Various methods of seed germination used for toxicity testing, their merits and demerits, and the influence of test medium and duration on test results were also covered. An overview on the advantages of using *O. sativa* as a test species in bioassyas has also been given. A literature review on phytotoxicity data revealed that there exists insufficient information on *O. sativa* from an ecotoxicological perspective.

In order to generate phytotoxicity data on the effect of some selected inorganic (cadmium, copper, and lead) and organic toxicants (phenol and sodium dodecyl sulfate) on *O. sativa*, its seeds were exposed to those toxicants and various toxicity estimates with regard to different morphometric endpoints were evaluated (see Chapter 2). Among the compounds selected cadmium, copper, phenol, and sodium dodecyl sulfate (SDS) belong to reference toxicants described by several agencies. The 'Jyothi' variety of *Oryza sativa* was used as the test species. The IC values (Inhibition Concentration – the concentration at which a specified level of inhibition of a specific biological response occurs) for each compound was computed for different durations of exposure. The results show that inorganic and organic compounds tested produced responses unique to

them. Cd was found to be the most toxic among metals, whereas phenol and SDS were found to be more or less similarly less toxic among organic toxicants. Sensitivity also varied with regard to morphological endpoints and the test durations considered. This chapter also covers the combined effects of cadmium and phenol on *O. sativa*. The study on combined effects revealed that cadmium and phenol act antagonistically on root growth of *O. sativa*.

Chapter 3 deals with the development of a TIE (toxicity identification evaluation) protocol for aqueous sample with *O. sativa*. Since the TIE protocol involves several physicochemical manipulations, the test species used in TIE should be able to tolerate them. Hence, the first step in TIE protocol was to standardize the TIE protocol for the test species to find out its range of tolerance to TIE manipulations. The TIE manipulations involved EDTA addition, oxidant reduction test, pH change, graduated pH test, filtration, solid phase extraction (SPE), aeration etc. The results indicated that *O. sativa* could survive TIE manipulations well within the prescribed ranges recommended by USEPA for other organisms. The final sections of this chapter present the TIE performed on a chemical mixture using *O. sativa*. The same components (cadmium and phenol) used to prepare the chemical mixture in the study of combined effect was used for TIE. The TIE manipulations used were within the range of tolerance (as computed in the previous section) of the test species. The results indicated that the EDTA treatment and SPE were successful in characterising the presence of cationic metal (Cd) and non-polar organics (phenol) in the chemical mixture.

As rice belongs to emergent macrophytes, it grows well in sediment and hence, this plant can be used as an excellent tool in sediment toxicity assessment. Chapter 4 presents the use of *O. sativa* in sediment toxicity tests. This chapter begins by providing an overview on Indian scenario of sediment quality assessment with special reference to knowledge gap in sediment toxicity data especially from Eloor-Edayar, an industrially dense region of Kerala situated at the lower reaches of the River Periyar. In order to assess the usefulness of *O. sativa* as a tool in sediment monitoring, sediment toxicity tests were performed with this test species using sediments collected from the River Periyar. The sediment samples were collected from three stations of the River Periyar during three seasons (monsoon, post-monsoon, pre-monsoon of 2013-2014). The station 1 represented relatively unpolluted area (upstream of Manappuram, Aluva) upstream of the industrial hotspot, the second and third samples were from regions of Eloor, an industrial hotspot of Kerala. The main purpose of this investigation was to identify a toxic hotspot so that the samples collected from there can further be used in the validation of sediment TIE (Chapter 5). Some sediment chemistry parameters were also investigated to provide a general picture of the nature of toxicity. Various biological parameters of *O. sativa* were assessed. The results revealed that none of the physicochemical variables from station 2 could be correlated with the cause of toxicity. However, the samples from station 3 showed a good negative correlation with electrical conductivity, suggesting the possibility of salinity ingression as the cause of toxicity. The sediment toxicity tests were repeated with the post-monsoon sample using a salinity tolerant

rice variety Vyttila-6 to rule out the possibility of salinity as the cause of growth inhibition.The growth of both the varieties (Jyothi and Vyttila-6) was highly inhibited in sediments from stations 2 and 3, indicating that salinity is not the main cause of growth inhibition.

The sediment elutriate tests were also carried out to check the possibility of toxicity due to resuspension of sediments (likely be caused by activities like dredging). Only the elutriate from station 2 of monsoon sample was found to be toxic to *O. sativa*.

Chapter 5 deals with sediment toxicity identification evaluation. The sediment TIE manipulations slightly differ from those followed with liquid samples and hence, the sediment TIE protocols need to be standardized for the test species. The sediment TIE manipulations involved treating the sediment samples with zeolite (to characterise ammonia), cation exchange resin (to characterise cationic metals), sulfide (to characterize metals), and charcoal (to characterize organic toxicants).

The earlier sections of chapter 5 focus on finding out the range of different chemical manipulations involved in TIE that the test species tolerates. For this purpose, the test species was grown in control sediment (prepared as per OECD guideline) with and without different concentrations of substances used as TIE treatments. Among various growth parameters observed, shoot length was not significantly affected by the TIE manipulations except for the sulfide addition test. The charcoal addition was hormetic (stimulatory) whereas CER and zeolite treatments were toxic root growth.

The final sections of this chapter presents the TIE with filed collected

sediment samples. The sediment samples were collected from one of the sites found to be toxic in sediment toxicity tests (see Chapter 4). A prominent reduction in toxicity was observed in manipulations with both sulfide and charcoal. However, the treatment with cation exchange resin, though showed a slight reduction in toxicity, did not yield significant improvement in growth. One probable reason might be the non-specific adsorption of ammonia cations or some other volatile compounds on to the cation exchange resin. To rule out this effect, the cation exchange resin treatment was repeated with dried sediment samples followed by sediment bioassay which resulted in significant reduction in toxicity. Thus, the results from both sulfide addition and cation exchange resin addition suggested the possibility of metals as the cause of phytotoxicity. The charcoal amendment reduced the toxicity significantly suggesting the involvement of organic toxicants. However, further analyses were focused on metals only. To confirm the presence of metals, the dried sediment samples were analysed for available metals. The available metal contents were correlated with the growth parameters and a significant linear relationship (negative correlation) with cadmium concentration and growth parameters confirmed the involvement of Cd as a toxicant. Manganese, showed a positive correlation with the growth parameters as it inhibits the cadmium toxicity by competing with it. Total Cd content extracted from the resin added to the dried sediment was found to be greater than the published sediment quality guidelines. In short, the sediment in the industrial belt of Periyar appears to be contaminated with metals and organic compounds at phytotoxic levels.

The test species *O. sativa* seems to be an ideal choice for toxicity tests. Further ecotoxicological investigations with *O. sativa* are warranted as this species lacks toxicity estimates for a vast number of compounds. Studies with salt tolerant rice species could also be undertaken to assess their utility by comparing them with other estuarine test species traditionally used in sediment toxicity tests. It is worth applying the latest advancements in TIE on this species. The only limitation of this work is that the extrapolatability of results from toxicity tests with *O. sativa* to the species of taxa other than monocot is relatively less. Future investigations similar to those presented here with other plant taxa is mandatory to establish its usefulness to the entire plant kingdom. The sediment TIE presented here is the first of its kind from the River Periyar.

Appendices

Appendix A

Chapter 2

A.1 Summary statistics for dose-response relationship of selected inorganic and organic toxicants with various morphometric endpoints of *O. sativa*.

Table A1. 1: Summary statistics for dose-response curve of root length at 96-h.

Compound (model used)	Model Parameters	Estimate	Std. Error	*t*-value	*p*-value
Cd (LL.3)[1]	*b*	2.13	0.28	7.6	<0.001
	d	24.04	0.91	26.3	<0.001
	e	5.05	0.35	14.25	<0.001
Cu (CRS.4a)[2*]	*b*	2.17	0.29	7.42	<0.001
	d	18.85	2.1	8.96	<0.001
	e	5.07	1.31	3.86	<0.001
	f	7.74	8.42	0.92	0.369
Pb (LL.3)[3]	*b*	2.7	0.86	3.13	<0.001
	d	24.79	1.51	16.43	<0.001
	e	43.66	5.99	7.29	<0.001
Phenol (LL.3)[4]	*b*	2.15	0.28	7.76	<0.001
	d	30.04	0.99	30.21	<0.001
	e	186.2	14.66	12.7	<0.001
SDS (BC.4)[5]	*b*	3.28	0.56	5.88	<0.001
	d	32.69	2.29	14.28	<0.001
	e	121.16	15.23	7.95	<0.001
	f	0.177	0.085	2.071	0.054

[1,2,3,4,5] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 1.592096 (12), 0.4893604(20), 3.745701 (18), 2.257647 (18), and 4.156704 (17), respectively. LL.3, CRS.4a, BC.4 denote log-logistic (3 parameter), Cedergreen-Ritz-Streibig modified log-logistic (for hormesis) with the lower limit equal to 0, and Brain-Cousens modified log-logistic (for describing u-shaped hormesis) models, respectively. For log-logistic model, the parameters *b*, *c*, *d*, and *e* represent slope, lower limit, upper limit, and IC_{50} (or EC/LC_{50}), respectively. For hormetic models, *f*, *c*, and *d* denote the size of hormesis, lower limit and upper limits, respectively, whereas *b* and *b* have no direct interpretations (Cedergreen et al., 2007; Ritz et al., 2015).

* = α set to 1.

Table A1. 2: Summary statistics for dose-response curve of root length at 72-h.

Compound (model used)	Model Parameters	Estimate	Std. Error	*t*-value	*p*-value
Cd (LL.3)[1]	*b*	2.42	0.40	6.07	<0.001
	d	12.50	0.62	20.14	<0.001
	e	7.61	0.67	11.36	<0.001
Cu (BC.4)[2 t]	*b*	2.69	0.39	6.82	<0.001
	d	8.69	1.07	8.14	<0.001
	e	6.10	1.60	3.83	<0.001
	f	1.44	0.99	1.46	0.1603
Pb (LL.3)[3]	*b*	3.30	0.96	3.44	<0.001
	d	12.12	0.52	23.36	<0.001
	e	49.69	4.42	11.24	<0.001
Phenol (LL.3)[4]	*b*	1.36	0.27	4.99	<0.001
	d	17.04	1.33	12.82	<0.001
	e	103.12	21.73	4.75	<0.001
SDS (LL.3)[5]	*b*	3.34	0.96	3.46	<0.001
	d	20.40	1.24	16.43	<0.001
	e	152.38	18.92	8.06	<0.001

[1,2,3,4,5] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 1.135418 (12), 0.8363724 (20), 1.446469 (18), 2.318299 (18), and 3.398278 (18), respectively. LL.3, CRS.4c, BC.4 denote log-logistic (3 parameter), Cedergreen-Ritz-Streibig modified log-logistic (for hormesis) with the lower limit equal to 0, and Brain-Cousens modified log-logistic (for describing u-shaped hormesis) models, respectively. For log-logistic model, the parameters b, c, d, and e represent slope, lower limit, upper limit, and IC_{50} (or EC/LC_{50}), respectively. For hormetic models, f, c, and d denote the size of hormesis, lower limit and upper limits, respectively, whereas b and b have no direct interpretations (Cedergreen et al., 2007; Ritz et al., 2015).

t = Box cox transformation applied.

Table A1. 3: Summary statistics for dose-response curve of shoot length at 96-h.

Compound (model used)	Model Parameters	Estimate	Std. Error	*t*-value	*p*-value
Cd (LL.3)[1]	*b*	1.92	0.40	4.85	<0.001
	d	13.47	0.66	20.50	<0.001
	e	21.46	2.44	8.80	<0.001
Cu (CRS.4c)[2*]	*b*	1.71	0.54	3.19	<0.001
	d	7.76	0.95	8.21	<0.001
	e	29.99	5.17	5.80	<0.001
	f	10.22	2.77	3.69	0.0014
Pb (BC.4)[3]	*b*	1.64	0.15	10.74	<0.001
	d	11.18	1.11	10.06	<0.001
	e	36.05	17.96	2.01	0.06
	f	0.41	0.30	1.36	0.19
Phenol (LL.3)[4 t]	*b*	14.11	2.14	6.61	<0.001
	d	18.73	1.19	15.68	<0.001
	e	487.56	5.05	96.62	<0.001
SDS (LL.3)[5]	*b*	2.37	0.61	3.90	<0.001
	d	20.74	1.07	19.47	<0.001
	e	434.81	61.77	7.04	<0.001

[1,2,3,4,5] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 1.457161 (18), 1.724578 (20), 0.162682 (17), 0.002681473 (18), and 3.11635(18), respectively. t = Box cox transformation applied. LL.3, CRS.4c, BC.4 denote log-logistic (3 parameter), Cedergreen-Ritz-Streibig modified log-logistic (for hormesis) with the lower limit equal to 0, and Brain-Cousens modified log-logistic (for describing u-shaped hormesis) models, respectively. For log-logistic model, the parameters b, c, d, and e represent slope, lower limit, upper limit, and IC_{50} (or EC/LC_{50}), respectively. For hormetic models, f, c, and d denote the size of hormesis, lower limit and upper limits, respectively, whereas b and b have no direct interpretations (Cedergreen et al., 2007; Ritz et al., 2015). * = α set to 0.25.

Table A1. 4: Summary statistics for dose-response curve of shoot length at 72-h.

Compound (model used)	**Model Parameters**	**Estimate**	**Std. Error**	***t*-value**	***p*-value**
Cd (LL.3)[1]	*b*	1.210	0.300	4.000	<0.001
	d	4.000	0.330	12.100	<0.001
	e	29.520	7.760	3.810	<0.001
Cu	NR	-	-	-	-
Pb	NR	-	-	-	-
Phenol (LL.3)[2 t]	*b*	2.880	1.070	2.700	<0.001
	d	6.880	0.440	15.610	<0.001
	e	433.27	31.26	13.86	<0.001
SDS (LL.3)[3]	*b*	4.460	1.110	4.020	<0.001
	d	7.390	0.410	18.250	<0.001
	e	524.46	41.846	12.533	<0.001

[1,2,3] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 0.7091784 (18), 0.2921413 (18), and, 0.3023425 (18), respectively. t = Box cox transformation applied. LL.3, CRS.4a, BC.4 denote log-logistic (3 parameter), Cedergreen-Ritz-Streibig modified log-logistic (for hormesis) with the lower limit equal to 0, and Brain-Cousens modified log-logistic (for describing u-shaped hormesis) models, respectively. For log-logistic model, the parameters b, c, d, and e represent slope, lower limit, upper limit, and IC_{50} (or EC/LC_{50}), respectively. For hormetic models, f, c, and d denote the size of hormesis, lower limit and upper limits, respectively, whereas b and b have no direct interpretations (Cedergreen et al., 2007; Ritz et al., 2015). NR = No significant dose-response relationship.

Table A1. 5: Summary statistics for dose-response curve of seedling length at 96-h.

Compound (model used)	Model parameters	Estimate	Std. Error	*t*-value	*p*-value
Cd (LL.3)[1]	*b*	1.43	0.12	11.54	<0.001
	d	37.52	1.16	32.22	<0.001
	e	8.14	0.63	13.00	<0.001
Cu (CRS.4a)[2*]	*b*	1.03	0.31	3.36	<0.001
	d	28.50	1.62	17.61	<0.001
	e	5.01	4.52	1.11	0.28
	f	27.78	27.04	1.03	0.317
Pb (LL.3)[3]	*b*	1.98	0.30	6.68	<0.001
	d	38.19	1.24	30.75	<0.001
	e	61.53	5.26	11.70	<0.001
Phenol (LL.3)[4]	*b*	2.67	0.51	5.24	<0.001
	d	47.78	1.60	29.77	<0.001
	e	301.54	23.89	12.62	<0.001
SDS (LL.3)[5]	*b*	2.45	0.51	4.80	<0.001
	d	58.08	2.69	21.62	<0.001
	e	217.41	23.78	9.14	<0.001

[1,2,3,4,5] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 2.047235 (18), 3.429985 (20), 3.296174 (18), 4.484318 (18), and 7.341984 (18), respectively. LL.3, CRS.4a, BC.4 denote log-logistic (3 parameter), Cedergreen-Ritz-Streibig modified log-logistic (for hormesis) with the lower limit equal to 0, and Brain-Cousens modified log-logistic (for describing u-shaped hormesis) models, respectively. For log-logistic model, the parameters *b*, *c*, *d*, and *e* represent slope, lower limit, upper limit, and IC_{50} (or EC/LC_{50}), respectively. For hormetic models, *f*, *c*, and *d* denote the size of hormesis, lower limit and upper limits, respectively, whereas *b* and *b* have no direct interpretations (Cedergreen et al., 2007; Ritz et al., 2015).

* = α set to 1.

Table A1. 6: Summary statistics for dose-response curve of seedling length at 72-h.

Compound (model used)	Model Parameters	Estimate	Std. Error	*t*-value	*p*-value
Cd (LL.3)[1]	*b*	1.88	0.19	9.7	<0.001
	d	16.47	0.52	31.77	<0.001
	e	9.66	0.64	15.16	<0.001
Cu (BC.4)[2]	*b*	1.97	0.33	6	<0.001
	d	13.25	1.28	10.32	<0.001
	e	5.45	2.45	2.22	0.04
	f	2.24	2.00	1.118	0.277
Pb (LL.3)[3]	*b*	1.81	0.32	5.64	<0.001
	d	16.55	0.64	25.93	<0.001
	e	70.66	7.53	9.39	<0.001
Phenol (LL.3)[4]	*b*	1.33	0.31	4.28	<0.001
	d	24.22	2.04	11.86	<0.001
	e	151.33	36.68	4.13	<0.001
SDS (LL.3)[5]	*b*	2.39	0.32	7.58	<0.001
	d	27.13	2.66	10.21	<0.001
	e	218.17	33.476	6.52	<0.001

[1,2,3,4,5] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 0.9645118 (18), 2.659378 (20), 1.672838 (18), 3.432064 (18), and 0.2659803 (18), respectively. LL.3, CRS.4a, BC.4 denote log-logistic (3 parameter), Cedergreen-Ritz-Streibig modified log-logistic (for hormesis) with the lower limit equal to 0, and Brain-Cousens modified log-logistic (for describing u-shaped hormesis) models, respectively. For log-logistic model, the parameters b, c, d, and e represent slope, lower limit, upper limit, and IC_{50} (or EC/LC_{50}), respectively. For hormetic models, f, c, and d denote the size of hormesis, lower limit and upper limits, respectively, whereas b and b have no direct interpretations (Cedergreen et al., 2007; Ritz et al., 2015).

Table A1. 7: Summary statistics for dose-response curve of seed germination at 96-h.

Compound (model used)	Model Parameters	Estimate	Std. Error	*t*-value	*p*-value
Cd (LL.2)	*b*	5.8	1.136	5.108	<0.001
	e	18.62	1.23	15.14	<0.001
Cu (LL.3)^{t}	*b*	4.097	0.93	4.403	<0.001
	d	0.962	0.015	65.7	<0.001
	e	24.08	2.046	11.77	<0.001
Pb (LL.2)	*b*	2.821	0.493	5.72	<0.001
	e	120.7	11.56	10.44	<0.001
Phenol (W1.3)	*b*	13.18	39.32	0.335	0.738
	d	0.993	0.007	152.8	<0.001
	e	541.4	129.4	4.184	<0.001
SDS (W1.3)	*b*	8.74	16.78	0.521	<0.001
	d	0.967	0.015	63.01	<0.001
	e	501.8	14.63	34.29	<0.001

LL.2, LL.3, and W1.3 denote log-logistic (2 parameter), log-logistic (3 parameter), and Weibull (3 parameter) models, respectively. For log-logistic model, the parameters *b*, *c*, *d*, and *e* represent slope, lower limit, upper limit, and IC_{50} (or EC/LC_{50}), respectively. For hormetic models, *f*, *c*, and *d* denote the size of hormesis, lower limit and upper limits, respectively, whereas *b* and *b* have no direct interpretations (Cedergreen et al., 2007; Ritz et al., 2015). t = Box cox transformation applied.

Table A1. 8: Summary statistics for dose-response curve of seed germination at 72-h.

Compound (model used)	**Model Parameters**	**Estimate**	**Std. Error**	***t*-value**	***p*-value**
Cd (LL.2)	*b*	17.48	79.57	0.220	0.8261
	e	15.00	0.31	47.865	<0.001
Cu (LL.3)	*b*	2.92	0.92	3.170	0.0015
	d	0.94	0.02	50.500	<0.001
	e	30.10	3.68	8.170	<0.001
Pb (W1.3)	*b*	3.02	0.72	4.200	<0.001
	d	0.98	0.01	65.657	<0.001
	e	105.85	8.40	12.606	<0.001
Phenol (W1.3)	*b*	8.74	16.78	0.521	0.6025
	d	0.97	0.02	63.011	<0.001
	e	501.76	14.63	34.289	<0.001
SDS (LL.3)	*b*	6.45	3.24	1.992	0.0464
	d	0.94	0.02	41.341	<0.001
	e	441.37	41.64	10.601	<0.001

LL.2, LL.3, and W1.3 denote log-logistic (2 parameter), log-logistic (3 parameter), and Weibull (3 parameter) models, respectively. For log-logistic model, the parameters b, c, d, and e represent slope, lower limit, upper limit, and IC_{50} (or EC/LC_{50}), respectively. For hormetic models, f, c, and d denote the size of hormesis, lower limit and upper limits, respectively, whereas b and b have no direct interpretations (Cedergreen et al., 2007; Ritz et al., 2015). t = Box cox transformation applied.

A.2 ANOVA summary for inorganic and organic toxicants.

Table A2. 1: ANOVA for the effect of selected inorganic and organic toxicants on root length of *O. sativa* at 96-h.

Cd (96-h)						
	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Concentration	4	1175.6	293.91	104.7	<0.001	***
Residuals	10	28.1	2.81			
Cu (96-h)						
Concentration	7	1652.6	236.1	26.84	<0.001	***
Residuals	16	140.7	8.8			
Pb (96-h)						
Concentration	6	2199.6	366.6	22.64	<0.001	***
Residuals	14	226.7	16.2			
Phenol (96-h)						
Concentration	6	3001.1	500.2	95.19	<0.001	***
Residuals	14	73.6	5.3			
SDS (96-h)						
Concentration	6	5501	916.9	44.83	<0.001	***
Residuals	14	286	20.5			

Table A2. 2: ANOVA for the effect of selected inorganic and organic toxicants on root length of *O. sativa* at 72-h.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Cd (72-h)						
Concentration	4	337.9	84.46	58.71	<0.001	***
Residuals	10	14.4	1.44			
Cu (72-h)						
Concentration	7	345.3	49.32	8.394	<0.001	***
Residuals	16	94	5.88			
Pb (72-h)						
Concentration	6	541.1	90.18	37.37	<0.001	***
Residuals	14	33.8	2.41			
Phenol (72-h)						
Concentration	6	782	130.3	21.02	<0.001	***
Residuals	14	86.8	6.2			
SDS (72-h)						
Concentration	6	1638.4	273.07	21.54	<0.001	***
Residuals	14	177.5	12.68			

Table A2. 3: ANOVA for the effect of selected inorganic and organic toxicants on shoot length of *O. sativa* at 96-h.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Cd (96-h)						
Concentration	6	502.8	83.79	35.91	<0.001	***
Residuals	14	32.7	2.33			
Cu (96-h)						
Concentration	7	113.6	16.228	7.469	<0.001	***
Residuals	16	34.76	2.173			
Pb (96-h)						
Concentration	6	201.44	33.57	8.154	<0.001	***
Residuals	14	57.65	4.12			
Phenol (96-h)						
Concentration	6	1156	192.66	23.81	<0.001	***
Residuals	14	113.3	8.09			
SDS (96-h)						
Concentration	6	1089	181.5	19.94	<0.001	***
Residuals	14	127.4	9.1			

Table A2. 4: ANOVA for the effect of selected inorganic and organic toxicants on shoot length of *O. sativa* at 72-h.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Cd (72-h)						
Concentration	6	29.826	4.971	9.171	<0.001	***
Residuals	14	7.589	0.542			
Cu (72-h)						
Concentration	7	1.11	0.159	0.05	1	
Residuals	16	51.12	3.195			
Pb (72-h)						
Concentration	6	5.684	0.9474	1.963	0.14	
Residuals	14	6.755	0.4825			
Phenol (72)						
Concentration	6	158.11	26.352	19.22	<0.001	***
Residuals	14	19.19	1.371			
SDS (72-H)						
Concentration	6	142.88	23.813	13.18	<0.001	***
Residuals	14	25.29	1.807			

Table A2. 5: ANOVA for the effect of selected inorganic and organic toxicants on seedling length of *O. sativa* at 96-h.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**(Pr(>*F*)**	**Sig.**
Cd (96-h)						
Concentration	6	3568	594.6	125.6	<0.001	***
Residuals	14	66	4.7			
Cu (96-h)						
Concentration	7	1954.8	279.26	22.18	<0.001	***
Residuals	16	201.4	12.59			
Pb (96-h)						
Concentration	6	3452	575.4	47.15	<0.001	***
Residuals	14	171	12.2			
Phenol (96-h)						
Concentration	6	7198	1199.6	58.53	<0.001	***
Residuals	14	287	20.5			
SDS (96-h)						
Concentration	6	10842	1807.1	37.01	<0.001	***
Residuals	14	684	48.8			

Table A2. 6: ANOVA for the effect of selected inorganic and organic toxicants on seedling length of *O. sativa* at 72-h.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Cd (72-h)						
Concentration	6	761.2	126.86	142	<0.001	***
Residuals	14	12.5	0.89			
Cu (72-h)						
Concentration	7	502.4	71.77	8.429	<0.001	***
Residuals	16	136.2	8.51			
Pb (72-h)						
Concentration	6	568.8	94.8	28.52	<0.001	***
Residuals	14	46.5	3.32			
Phenol (72)						
Concentration	6	1563.8	260.64	22.4	<0.001	***
Residuals	14	162.9	11.64			
SDS (72-H)						
Concentration	6	2594.9	432.5	20.31	<0.001	***
Residuals	14	298.2	21.3			

Table A2. 7: ANOVA for the effect of selected inorganic and organic toxicants on seed germination of *O. sativa* at 96-h.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Cd (96-h)						
Concentration	5	329.6	65.92	131.8	<0.001	***
Residuals	12	6	0.5			
Cu (96-h)						
Concentration	7	138	19.71	39.43	<0.001	***
Residuals	16	8	0.5			
Pb (96-h)						
Concentration	6	159.14	26.524	55.7	<0.001	***
Residuals	14	6.67	0.476			
Phenol (96-h)						
Concentration	6	251.14	41.86	67.61	<0.001	***
Residuals	14	8.67	0.62			
SDS (96-h)						
Concentration	6	285.1	47.52	55.44	<0.001	***
Residuals	14	12	0.86			

Table A2. 8: ANOVA for the effect of selected inorganic and organic toxicants on seed germination of *O. sativa* at 72-h.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Cd (72-h)						
Concentration	4	229.07	57.27	66.08	<0.001	***
Residuals	10	8.67	0.87			
Cu (72-h)						
Concentration	7	67.17	9.595	5.355	0.00264	**
Residuals	16	28.67	1.792			
Pb (72-h)						
Concentration	6	263.81	43.97	40.15	<0.001	***
Residuals	14	15.33	1.1			
Phenol (72-h)						
Concentration	6	285.1	47.52	55.44	<0.001	***
Residuals	14	12	0.86			
SDS (72-h)						
Concentration	6	277.14	46.19	26.22	<0.001	***
Residuals	14	24.67	1.76			

A.3 Post hoc test for the effect of selected inorganic and organic toxicants on root length of *O. sativa*. (concentrations in mg/L).

Table A3. 1: Dunnet's multiple comparison (one tailed) for the effect of $CdCl_2$ on root length of *O. sativa*.

$CdCl_2$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
3.75 - 0 >= 0	-8.182	1.368	-5.98	0.000222	***
7.5 - 0 >= 0	-17.093	1.368	-12.49	<0.001	***
15 - 0 >= 0	-21.236	1.368	-15.52	<0.001	***
30 - 0 >= 0	-24.007	1.368	-17.55	<0.001	***
$CdCl_2$ (72-h)	**Estimate**	**Std. Error**	***t*-value**		**Sig.**
3.75 - 0 >= 0	-1.729	0.9794	-1.765	0.147	
7.5 - 0 >= 0	-6.2861	0.9794	-6.419	<0.001	***
15 - 0 >= 0	-10.0893	0.9794	-10.302	<0.001	***
30 - 0 >= 0	-12.4525	0.9794	-12.715	<0.001	***

Table A3. 2: Dunnet's multiple comparison (one tailed) for the effect of $CdCl_2$ on shoot length of *O. sativa*.

$CdCl_2$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
3.75 - 0 >= 0	-0.9407	1.2473	-0.754	0.56915	
7.5 - 0 >= 0	-1.1691	1.2473	-0.937	0.48537	
15 - 0 >= 0	-4.3016	1.2473	-3.449	0.00919	**
30 - 0 >= 0	-9.5662	1.2473	-7.669	< 0.001	***
60 - 0 >= 0	-11.4596	1.2473	-9.187	< 0.001	***
120 - 0 >= 0	-12.1694	1.2473	-9.756	< 0.001	***
$CdCl_2$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
3.75 - 0 >= 0	0.05513	0.60115	0.092	0.88028	
7.5 - 0 >= 0	0.04767	0.60115	0.079	0.87734	
15 - 0 >= 0	-1.3806	0.60115	-2.297	0.07551	.
30 - 0 >= 0	-1.93253	0.60115	-3.215	0.01425	*
60 - 0 >= 0	-2.45143	0.60115	-4.078	0.00262	**
120 - 0 >= 0	-3.0013	0.60115	-4.993	< 0.001	***

Table A3. 3: Dunnet's multiple comparison (one tailed) for the effect of $CdCl_2$ on seedling length of *O. sativa*.

$CdCl_2$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
3.75 - 0 >= 0	-9.123	1.776	-5.136	0.000293	***
7.5 - 0 >= 0	-18.262	1.776	-10.281	< 0.001	***
15 - 0 >= 0	-25.387	1.776	-14.293	< 0.001	***
30 - 0 >= 0	-33.574	1.776	-18.901	< 0.001	***
60 - 0 >= 0	-35.467	1.776	-19.967	< 0.001	***
120 - 0 >= 0	-36.177	1.776	-20.367	< 0.001	***
$CdCl_2$ (72-h).	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
3.75 - 0 >= 0	-1.6738	0.7717	-2.169	0.0935	.
7.5 - 0 >= 0	-6.2384	0.7717	-8.084	<0.001	***
15 - 0 >= 0	-11.4699	0.7717	-14.862	<0.001	***
30 - 0 >= 0	-14.385	0.7717	-18.64	<0.001	***
60 - 0 >= 0	-14.9039	0.7717	-19.312	<0.001	***
120 - 0 >= 0	-15.4538	0.7717	-20.025	<0.001	***

Table A3. 4: Dunnet's multiple comparison (one tailed) for the effect of $CdCl_2$ on seed germination of *O. sativa*.

$CdCl_2$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
3.75 - 0 >= 0	0.6667	0.5774	1.155	0.9876	
7.5 - 0 >= 0	0.6667	0.5774	1.155	0.9877	
15 - 0 >= 0	-1.6667	0.5774	-2.887	0.0258	*
30 - 0 >= 0	-8.6667	0.5774	-15.011	<0.001	***
60 - 0 >= 0	-9.3333	0.5774	-16.166	<0.001	***
$CdCl_2$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	
3.75 - 0 >= 0	0.6667	0.7601	0.877	0.9646	
7.5 - 0 >= 0	0.6667	0.7601	0.877	0.9646	
15 - 0 >= 0	-4.3333	0.7601	-5.701	0.0003	***
30 - 0 >= 0	-9.3333	0.7601	-12.279	< 0.001	***

Table A3. 5: Dunnet's multiple comparison (one tailed) for the effect of $CuSO_4$ on root length of *O. sativa*.

$CuSO_4$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.5 - 0 >= 0	-3.3436	2.4215	-1.381	0.319	
1 - 0 >= 0	3.6461	2.4215	1.506	0.9977	
2 - 0 >= 0	-0.2974	2.4215	-0.123	0.8419	
4 - 0 >= 0	-5.6109	2.4215	-2.317	0.0771	.
8 - 0 >= 0	-12.1927	2.4215	-5.035	<0.001	***
16 - 0 >= 0	-18.5831	2.4215	-7.674	<0.001	***
32 - 0 >= 0	-19.9303	2.4215	-8.231	<0.001	***
$CuSO_4$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.5 - 0 >= 0	-1.376	1.979	-0.695	0.62369	
1 - 0 >= 0	0.311	1.979	0.157	0.90976	
2 - 0 >= 0	1.481	1.979	0.748	0.97887	
4 - 0 >= 0	1.71	1.979	0.864	0.98463	
8 - 0 >= 0	-2.686	1.979	-1.357	0.32831	
16 - 0 >= 0	-7.948	1.979	-4.016	0.00299	**
32 - 0 >= 0	-8.617	1.979	-4.354	0.00142	**

Table A3. 6: Dunnet's multiple comparison (one tailed) for the effect of $CuSO_4$ on shoot length of *O. sativa*.

CuSO4 (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.5 - 0 >= 0	4.4419	1.2035	3.691	1	
1 - 0 >= 0	2.5215	1.2035	2.095	1	
2 - 0 >= 0	3.2328	1.2035	2.686	1	
4 - 0 >= 0	5.1468	1.2035	4.277	1	
8 - 0 >= 0	5.8203	1.2035	4.836	1	
16 - 0 >= 0	1.4891	1.2035	1.237	0.995	
32 - 0 >= 0	-0.4745	1.2035	-0.394	0.75	

Table A3. 7: Dunnet's multiple comparison (one tailed) for the effect of $CuSO_4$ on shoot length of *O. sativa*.

$CuSO_4$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.5 - 0 >= 0	0.059	1.45952	0.04	0.885	
1 - 0 >= 0	0.1735	1.45952	0.119	0.902	
2 - 0 >= 0	0.41083	1.45952	0.281	0.931	
4 - 0 >= 0	-0.38843	1.45952	-0.266	0.797	
8 - 0 >= 0	-0.10383	1.45952	-0.071	0.856	
16 - 0 >= 0	0.12703	1.45952	0.087	0.895	
32 - 0 >= 0	0.09193	1.45952	0.063	0.89	

Table A3. 8: Dunnet's multiple comparison (one tailed) for the effect of $CuSO_4$ on seedling length of *O. sativa*.

$CuSO_4$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.5 - 0 >= 0	1.0982	2.8972	0.379	0.9455	
1 - 0 >= 0	6.1676	2.8972	2.129	0.9997	
2 - 0 >= 0	2.9353	2.8972	1.013	0.99	
4 - 0 >= 0	-0.4641	2.8972	-0.16	0.8308	
8 - 0 >= 0	-6.3724	2.8972	-2.2	0.0945	.
16 - 0 >= 0	-17.094	2.8972	-5.9	<0.001	***
32 - 0 >= 0	-20.4048	2.8972	-7.043	<0.001	***
$CuSO_4$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.5 - 0 >= 0	0.3141	2.3824	0.132	0.9047	
1 - 0 >= 0	2.2252	2.3824	0.934	0.98737	
2 - 0 >= 0	1.7086	2.3824	0.717	0.97697	
4 - 0 >= 0	1.2336	2.3824	0.518	0.96128	
8 - 0 >= 0	-3.3537	2.3824	-1.408	0.30853	
16 - 0 >= 0	-8.7546	2.3824	-3.675	0.00559	**
32 - 0 >= 0	-10.2877	2.3824	-4.318	0.00147	**

Table A3. 9: Dunnet's multiple comparison (one tailed) for the effect of $CuSO_4$ on seed germination of *O. sativa*.

$CuSO_4$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.5 - 0 >= 0	4.25E-15	5.77E-01	0	0.875	
1 - 0 >= 0	3.33E-01	5.77E-01	0.577	0.9667	
2 - 0 >= 0	3.33E-01	5.77E-01	0.577	0.9667	
4 - 0 >= 0	6.67E-01	5.77E-01	1.155	0.9934	
8 - 0 >= 0	3.33E-01	5.77E-01	0.577	0.9667	
16 - 0 >= 0	-1.33E+00	5.77E-01	-2.309	0.0782	.
32 - 0 >= 0	-7.00E+00	5.77E-01	-12.124	<0.001	***
$CuSO_4$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.5 - 0 >= 0	-8.32E-17	1.09E+00	0	0.875	
1 - 0 >= 0	8.18E-17	1.09E+00	0	0.875	
2 - 0 >= 0	-1.84E-16	1.09E+00	0	0.875	
4 - 0 >= 0	3.33E-01	1.09E+00	0.305	0.935	
8 - 0 >= 0	-7.40E-16	1.09E+00	0	0.875	
16 - 0 >= 0	-1.33E+00	1.09E+00	-1.22	0.385	
32 - 0 >= 0	-5.00E+00	1.09E+00	-4.575	<0.001	***

Table A3. 10: Dunnet's multiple comparison (one tailed) for the effect of $PbN(O_3)_2$ on root length of *O. sativa*.

$PbN(O_3)_2$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
6.25 - 0 >= 0	0.09957	3.28575	0.03	0.8651	
12.5 - 0 >= 0	-3.5546	3.28575	-1.082	0.42071	
25 - 0 >= 0	-5.4844	3.28575	-1.669	0.20263	
50 - 0 >= 0	-14.52757	3.28575	-4.421	0.00141	**
100 - 0 >= 0	-24.7056	3.28575	-7.519	< 0.001	***
200 - 0 >= 0	-25.3049	3.28575	-7.701	< 0.001	***

Table A3. 11: Dunnet's multiple comparison (one tailed) for the effect of $PbN(O_3)_2$ on root length of *O. sativa*.

$PbN(O_3)_2$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
6.25 - 0 >= 0	-1.082	1.268	-0.853	0.524	
12.5 - 0 >= 0	-1.041	1.268	-0.821	0.539	
25 - 0 >= 0	-2.363	1.268	-1.863	0.152	
50 - 0 >= 0	-6.547	1.268	-5.162	<0.001	***
100 - 0 >= 0	-12.287	1.268	-9.688	<0.001	***
200 - 0 >= 0	-12.879	1.268	-10.155	<0.001	***

Table A3. 12: Dunnet's multiple comparison (one tailed) for the effect of $PbN(O_3)_2$ on shoot length of *O. sativa*.

$PbN(O_3)_2$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
6.25 - 0 >= 0	0.5196	1.6568	0.314	0.9248	
12.5 - 0 >= 0	4.244	1.6568	2.562	0.99986	
25 - 0 >= 0	1.3619	1.6568	0.822	0.97811	
50 - 0 >= 0	0.2474	1.6568	0.149	0.89336	
100 - 0 >= 0	-2.8934	1.6568	-1.746	0.18143	
200 - 0 >= 0	-6.3021	1.6568	-3.804	0.00459	**
$PbN(O_3)_2$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
6.25 - 0 >= 0	0.4824	0.5672	0.85	0.98	
12.5 - 0 >= 0	0.6227	0.5672	1.098	0.99	
25 - 0 >= 0	0.5573	0.5672	0.983	0.986	
50 - 0 >= 0	0.7846	0.5672	1.383	0.995	
100 - 0 >= 0	0.9475	0.5672	1.671	0.998	
200 - 0 >= 0	-0.6989	0.5672	-1.232	0.356	

Table A3. 13: Dunnet's multiple comparison (one tailed) for the effect of $PbN(O_3)_2$ on seedling length of *O. sativa*.

$PbN(O_3)_2$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
6.25 - 0 >= 0	0.6192	2.8524	0.217	0.907	
12.5 - 0 >= 0	0.6894	2.8524	0.242	0.912	
25 - 0 >= 0	-4.1225	2.8524	-1.445	0.275	
50 - 0 >= 0	-14.2802	2.8524	-5.006	<0.001	***
100 - 0 >= 0	-27.599	2.8524	-9.676	<0.001	***
200 - 0 >= 0	-31.607	2.8524	-11.081	<0.001	***
$PbN(O_3)_2$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
6.25 - 0 >= 0	-0.5995	1.4887	-0.403	0.72168	
12.5 - 0 >= 0	-0.4183	1.4887	-0.281	0.76813	
25 - 0 >= 0	-1.8057	1.4887	-1.213	0.36461	
50 - 0 >= 0	-5.7627	1.4887	-3.871	0.00403	**
100 - 0 >= 0	-11.3394	1.4887	-7.617	<0.001	***
200 - 0 >= 0	-13.5782	1.4887	-9.121	<0.001	***

Table A3. 14: Dunnet's multiple comparison (one tailed) for the effect of $PbN(O_3)_2$ on seed germination of *O. sativa*.

$PbN(O_3)_2$ (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
6.25 - 0 >= 0	6.67E-01	5.63E-01	1.183	0.992	
12.5 - 0 >= 0	6.67E-01	5.63E-01	1.183	0.992	
25 - 0 >= 0	6.67E-01	5.63E-01	1.183	0.992	
50 - 0 >= 0	2.07E-15	5.63E-01	0	0.857	
100 - 0 >= 0	-3.67E+00	5.63E-01	-6.508	<1e-04	***
200 - 0 >= 0	-7.00E+00	5.63E-01	-12.424	<1e-04	***

Table A3. 15: Dunnet's multiple comparison (one tailed) for the effect of $PbN(O_3)_2$ on seed germination of *O. sativa*.

$PbN(O_3)_2$ (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
6.25 - 0 >= 0	7.79E-17	8.55E-01	0	0.857	
12.5 - 0 >= 0	3.33E-01	8.55E-01	0.39	0.937	
25 - 0 >= 0	-3.94E-16	8.55E-01	0	0.857	
50 - 0 >= 0	-1.00E+00	8.55E-01	-1.17	0.382	
100 - 0 >= 0	-5.33E+00	8.55E-01	-6.242	<1e-04	***
200 - 0 >= 0	-9.67E+00	8.55E-01	-11.313	<1e-04	***

Table A3. 16: Dunnet's multiple comparison (one tailed) for the effect of phenol on root length of *O. sativa*.

Phenol (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	-0.6002	1.8716	-0.321	0.753	
62.5 - 0 >= 0	-3.738	1.8716	-1.997	0.124	
125 - 0 >= 0	-9.7354	1.8716	-5.202	<0.001	***
250 - 0 >= 0	-18.5783	1.8716	-9.926	<0.001	***
500 - 0 >= 0	-28.7442	1.8716	-15.358	<0.001	***
1000 - 0 >= 0	-30.386	1.8716	-16.235	<0.001	***
Phenol (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	-3.447	2.033	-1.695	0.19552	
62.5 - 0 >= 0	-6.467	2.033	-3.18	0.01518	*
125 - 0 >= 0	-9.199	2.033	-4.524	0.00136	**
250 - 0 >= 0	-12.771	2.033	-6.281	<0.001	***
500 - 0 >= 0	-16.746	2.033	-8.236	<0.001	***
1000 - 0 >= 0	-17.332	2.033	-8.524	<0.001	***

Table A3. 17: Dunnet's multiple comparison (one tailed) for the effect of phenol on shoot length of *O. sativa*.

Phenol (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	-3.477	2.3228	-1.497	0.257	
62.5 - 0 >= 0	-2.8504	2.3228	-1.227	0.359	
125 - 0 >= 0	0.6533	2.3228	0.281	0.919	
250 - 0 >= 0	-1.8458	2.3228	-0.795	0.551	
500 - 0 >= 0	-13.0135	2.3228	-5.602	<0.001	***
1000 - 0 >= 0	-20.9121	2.3228	-9.003	<0.001	***
Phenol (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	-1.281	0.956	-1.34	0.314	
62.5 - 0 >= 0	-1.679	0.956	-1.757	0.179	
125 - 0 >= 0	-1.355	0.956	-1.418	0.285	
250 - 0 >= 0	-2.246	0.956	-2.349	0.069	.
500 - 0 >= 0	-6.334	0.956	-6.625	<0.001	***
1000 - 0 >= 0	-7.962	0.956	-8.328	<0.001	***

Table A3. 18: Dunnet's multiple comparison (one tailed) for the effect of phenol on seedling length of *O. sativa*.

Phenol (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	-4.077	3.696	-1.103	0.4113	
62.5 - 0 >= 0	-6.588	3.696	-1.782	0.1722	
125 - 0 >= 0	-9.082	3.696	-2.457	0.0573	.
250 - 0 >= 0	-20.424	3.696	-5.525	<0.001	***
500 - 0 >= 0	-41.758	3.696	-11.297	<0.001	***
1000 - 0 >= 0	-51.298	3.696	-13.878	<0.001	***

Table A3. 19: Dunnet's multiple comparison (one tailed) for the effect of phenol on seedling length of *O. sativa*.

Phenol (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	-4.728	2.785	-1.697	0.1949	
62.5 - 0 >= 0	-8.146	2.785	-2.925	0.0244	*
125 - 0 >= 0	-10.554	2.785	-3.79	0.0046	**
250 - 0 >= 0	-15.017	2.785	-5.392	<0.001	***
500 - 0 >= 0	-23.08	2.785	-8.287	<0.001	***
1000 - 0 >= 0	-25.294	2.785	-9.082	<0.001	***

Table A3. 20: Dunnet's multiple comparison (one tailed) for the effect of phenol on seed germination of *O. sativa*.

Phenol (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	1.31E-15	6.42E-01	0	0.85716	
62.5 - 0 >= 0	-3.33E-01	6.42E-01	-0.519	0.67365	
125 - 0 >= 0	1.42E-15	6.42E-01	0	0.85712	
250 - 0 >= 0	2.22E-15	6.42E-01	0	0.85715	
500 - 0 >= 0	-3.00E+00	6.42E-01	-4.67	0.00105	**
1000 - 0 >= 0	-1.00E+01	6.42E-01	-15.566	< 0.001	***
Phenol (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	-1.00E+00	7.56E-01	-1.323	0.32	
62.5 - 0 >= 0	-3.33E-01	7.56E-01	-0.441	0.706	
125 - 0 >= 0	1.18E-15	7.56E-01	0	0.857	
250 - 0 >= 0	-3.33E-01	7.56E-01	-0.441	0.706	
500 - 0 >= 0	-6.33E+00	7.56E-01	-8.378	<0.001	***
1000 - 0 >= 0	-1.00E+01	7.56E-01	-13.229	<0.001	***

Table A3. 21: Dunnet's multiple comparison (one tailed) for the effect of SDS on root length of *O. sativa*.

SDS (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	3.917	3.693	1.061	0.989	
62.5 - 0 >= 0	6.922	3.693	1.874	0.999	
125 - 0 >= 0	-7.44	3.693	-2.015	0.12	
250 - 0 >= 0	-25.859	3.693	-7.003	<0.001	***
500 - 0 >= 0	-32.641	3.693	-8.839	<0.001	***
1000 - 0 >= 0	-33.023	3.693	-8.943	<0.001	***
SDS (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	2.794	2.907	0.961	0.985	
62.5 - 0 >= 0	3.087	2.907	1.062	0.989	
125 - 0 >= 0	-5.648	2.907	-1.943	0.135	
250 - 0 >= 0	-14.262	2.907	-4.906	<0.001	***
500 - 0 >= 0	-17.878	2.907	-6.15	<0.001	***
1000 - 0 >= 0	-18.259	2.907	-6.281	<0.001	***

Table A3. 22: Dunnet's multiple comparison (one tailed) for the effect of SDS on shoot length of *O. sativa*.

SDS (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	2.1519	2.4634	0.874	0.9809	
62.5 - 0 >= 0	2.0756	2.4634	0.843	0.97924	
125 - 0 >= 0	0.2976	2.4634	0.121	0.88702	
250 - 0 >= 0	-4.832	2.4634	-1.962	0.13096	
500 - 0 >= 0	-8.7168	2.4634	-3.539	0.00768	**
1000 - 0 >= 0	-19.1932	2.4634	-7.791	< 0.001	***

Table A3. 23: Dunnet's multiple comparison (one tailed) for the effect of SDS on shoot length of *O. sativa*.

SDS (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	0.6736	1.0975	0.614	0.9628	
62.5 - 0 >= 0	0.056	1.0975	0.051	0.8704	
125 - 0 >= 0	-0.4281	1.0975	-0.39	0.7267	
250 - 0 >= 0	-1.6023	1.0975	-1.46	0.2697	
500 - 0 >= 0	-3.4082	1.0975	-3.106	0.0173	*
1000 - 0 >= 0	-7.3103	1.0975	-6.661	<0.001	***

Table A3. 24: Dunnet's multiple comparison (one tailed) for the effect of SDS on seedling length of *O. sativa*.

SDS (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	6.069	5.706	1.064	0.989	
62.5 - 0 >= 0	8.997	5.706	1.577	0.997	
125 - 0 >= 0	-7.143	5.706	-1.252	0.349	
250 - 0 >= 0	-30.691	5.706	-5.379	<0.001	***
500 - 0 >= 0	-41.358	5.706	-7.249	<0.001	***
1000 - 0 >= 0	-52.216	5.706	-9.152	<0.001	***
SDS (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	3.468	3.768	0.92	0.98315	
62.5 - 0 >= 0	3.143	3.768	0.834	0.97882	
125 - 0 >= 0	-6.076	3.768	-1.613	0.21971	
250 - 0 >= 0	-15.864	3.768	-4.21	0.00221	**
500 - 0 >= 0	-21.286	3.768	-5.649	< 0.001	***
1000 - 0 >= 0	-25.569	3.768	-6.786	< 0.001	***

Table A3. 25: Dunnet's multiple comparison (one tailed) for the effect of SDS on seed germination of *O. sativa* at 96-h.

SDS (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	-1.00E+00	7.56E-01	-1.323	0.321	
62.5 - 0 >= 0	-3.33E-01	7.56E-01	-0.441	0.706	
125 - 0 >= 0	1.18E-15	7.56E-01	0	0.857	
250 - 0 >= 0	-3.33E-01	7.56E-01	-0.441	0.706	
500 - 0 >= 0	-6.33E+00	7.56E-01	-8.378	<0.001	***
1000 - 0 >= 0	-1.00E+01	7.56E-01	-13.229	<0.001	***
SDS (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
31.25 - 0 >= 0	3.33E-01	1.08E+00	0.308	0.924	
62.5 - 0 >= 0	3.26E-15	1.08E+00	0	0.857	
125 - 0 >= 0	2.56E-15	1.08E+00	0	0.857	
250 - 0 >= 0	-3.33E-01	1.08E+00	-0.308	0.758	
500 - 0 >= 0	-6.33E+00	1.08E+00	-5.844	< 0.001	***
1000 - 0 >= 0	-9.33E+00	1.08E+00	-8.612	< 0.001	***

Appendix B

Chapter 3

B.1 Summary statistics for TIE with aqueous sample.

Table B1. 1: Summary statistics for dose-response curve of TIE manipulations (intrinsic toxicity) with *O. sativa* in aqueous samples.

Duration (model used)	Model Parameters	Estimate	Std. Error	*t*-value	*p*-value
Compound: EDTA; Endpoint: Root length					
96-h (CRS.4a)[1]	*b*	2.139	0.616	3.470	0.0029
	d	20.483	2.697	7.596	<0.001
	e	161.917	26.459	6.119	<0.001
	f	9.166	3.583	2.558	0.0204
72-h (LL.3)[2]	*b*	2.551	0.773	3.300	0.003985
	d	14.641	0.708	20.683	<0.001
	e	233.315	30.500	7.650	<0.001
Compound: STS; Endpoint: Root length					
96-h (BC.4)[3]	*b*	2.30E+00	3.32E-01	6.93E+00	<0.001
	d	1.95E+01	2.35E+00	8.29E+00	<0.001
	e	1.79E+03	4.55E+02	3.93E+00	0.0011
	f	1.93E-02	8.44E-03	2.29E+00	0.0349
72-h (BC.4)[4]	*b*	2.28E+00	2.85E-01	8.01E+00	<0.001
	d	8.72E+00	8.31E-01	1.05E+01	<0.001
	e	1.98E+03	4.41E+02	4.49E+00	<0.001
	f	6.45E-03	2.67E-03	2.42E+00	0.027266

[1,2,3,4] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 5.536972 (18), 2.323815 (18), 4.419495 (17), 1.606109 (17), respectively. LL.3, CRS.4a, BC.4 denote log-logistic (3 parameter), Cedergreen-Ritz-Streibig modified log-logistic (for hormesis) with the lower limit equal to 0, and Brain-Cousens modified log-logistic (for describing u-shaped hormesis) models, respectively.

Table B1. 2: Summary statistics for dose-response curve of TIE manipulations (intrinsic toxicity) with *O. sativa* in aqueous samples.

Compound: Methanol; Endpoint: Root length					
Duration (model used)	**Model Parameters**	**Estimate**	**Std. Error**	***t*-value**	***p*-value**
96-h (W2.3)[1]	*b*	-1.689	0.698	-2.421	0.02864
	d	32.631	3.535	9.230	<0.001
	e	1.384	0.352	3.936	0.00132
72-h (W2.3)[2]	*b*	-2.940	1.195	-2.461	<0.001
	d	16.720	1.308	12.780	<0.001
	e	1.101	0.162	6.799	<0.001

[1,2] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 9.191891 (15), 3.334416 (15,) respectively. W2.3 denotes Weibull (3 parameter) type 2, model.

Table B1. 3: Summary statistics for dose-response curve of TIE manipulations (intrinsic toxicity) with *O. sativa* in aqueous samples.

Duration (model used)	Model Parameters	Estimate	Std. Error	*t*-value	*p*-value
Compound: EDTA; Endpoint: Shoot					
96-h	NR				
72-h	NR				
Compound: STS; Endpoint: Shoot					
96-h (W2.3)[1 t]	*b*	-0.832	0.195	-4.255	<0.001
	d	14.149	0.430	32.893	<0.001
	e	5633.6	974.9	5.779	<0.001
72-h	NR				
Compound: Methanol; Endpoint: Shoot					
96-h (LL.3)[2]	*b*	2.058	0.611	3.369	<0.001
	d	21.232	1.771	11.988	<0.001
	e	1.454	0.261	5.5805	<0.001
72-h (W1.3)[3]	*b*	1.294	0.353	3.6658	<0.001
	d	10.103	0.798	12.6625	<0.001
	e	1.849	0.263	7.0321	<0.001

[1,2,3] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 5.348363 (18), 3.600186 (15), 1.394708 (15), respectively. LL.3, W1.3, and W2.3 denote log-logistic (3 parameter), Weibull (3 parameter) type 1, and Weibull (3 parameter) type 2 models, respectively. t = Boxcox transformation applied. NR = No significant dose-response relationship.

Table B1. 4: Summary statistics for dose-response curve of TIE manipulations (intrinsic toxicity) with *O. sativa* in aqueous samples.

Duration (model used)	**Model Parameters**	**Estimate**	**Std. Error**	***t*-value**	***p*-value**
Compound: EDTA; Endpoint: Seedling					
96-h (W2.3)[1]	*b*	-1.035	0.269	-3.851	<0.001
	d	44.923	1.698	26.452	<0.001
	e	236.255	36.529	6.468	<0.001
72-h (LL.3)[2]	*b*	1.905	0.560	3.399	<0.001
	d	20.981	0.852	24.627	<0.001
	e	360.152	52.264	6.891	<0.001
Compound: STS; Endpoint: Seedling					
96-h (BC.4)[3]	*b*	1.960E+00	2.180E-01	8.996E+00	<0.001
	d	3.280E+01	2.880E+00	1.140E+01	<0.001
	e	1.790E+03	5.130E+02	3.497E+00	<0.001
	f	2.620E-02	1.220E-02	2.147E+00	0.0465
72-h (BC.4)[4]	*b*	1.870E+00	1.600E-01	1.169E+01	<0.001
	d	1.300E+01	8.500E-01	1.524E+01	<0.001
	e	1.900E+03	4.320E+02	4.393E+00	<0.001
	f	9.250E-03	3.400E-03	2.720E+00	0.014542

[1,2,3,4] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 5.741539 (18), 2.74383 (18), 5.350916 (17), 1.628192 (17), respectively. LL.3, and W2.3, and BC.4 denote log-logistic (3 parameter) type 2, Brain-Cousens modified log-logistic (for describing u-shaped hormesis) models, respectively.

Table B1. 5: Summary statistics for dose-response curve of TIE manipulations (intrinsic toxicity) with *O. sativa* in aqueous samples.

Compound: Methanol; Endpoint: Seedling					
Duration (model used)	**Model Parameters**	**Estimate**	**Std. Error**	***t*-value**	***p*-value**
96-h (W2.3)[1]	*b*	-1.667	0.545	-3.062	7.91E-03
	d	52.948	4.643	11.403	<0.001
	e	1.297	0.265	4.891	<0.001
72-h (W2.3)[2]	*b*	-2.320	0.663	-3.500	0.0032
	d	25.938	1.684	15.404	<0.001
	e	1.125	0.145	7.782	<0.001

[1,2] = Models with the corresponding numbers have residual R-squared (and degrees of freedom) 11.75135 (15), 4.216673 (15), respectively. W2.3 denotes Weibull (3 parameter) type 2, model.

Table B1. 6: Summary statistics for dose-response curve of TIE manipulations (intrinsic toxicity) with *O. sativa* in aqueous samples.

Duration (model used)	**Model Parameters**	**Estimate**	**Std. Error**	***t*-value**	***p*-value**
Compound: EDTA; Endpoint: Germination					
96-h	NR				
72-h	NR				
Compound: STS; Endpoint: Germination					
96-h	NR				
72-h	NR				
Compound: Methanol; Endpoint: Germination					
96-h (W1.3)[1]	*b*	2.983	0.911	3.275	0.001055
	d	0.961	0.028	34.356	<0.001
	e	3.666	0.301	12.197	<0.001
72-h (W1.2)[2]	*b*	1.357	0.194	6.989	<0.001
	e	2.178	0.226	9.632	<0.001

W1.3 and W2.3 denote Weibull (3 parameter) type 1 and type 3, models respectively. NR = No significant dose-response relationship. NR = No significant dose-response relationship.

B.2 Intrinsic toxicity of TIE manipulation in aqueous sample: ANOVA summary for root.

Table B2. 1: ANOVA for the effect of TIE manipulations (intrinsic toxicity) on the root elongation of *O. sativa* in aqueous samples.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
EDTA (96 –hr)						
Concentration	6	1710.3	285.05	12.04	>0.001	***
Residuals	14	331.6	23.69			
EDTA (72-h)						
Concentration	6	382.4	63.73	11.31	>0.001	***
Residuals	14	78.9	5.63			
STS (96-h)						
Concentration	6	1697.9	282.98	13.23	>0.001	***
Residuals	14	299.5	21.39			
STS (72-h)						
Concentration	6	265.22	44.2	15.06	>0.001	***
Residuals	14	41.08	2.93			
Methanol (96-h)						
Concentration	5	3001	600.2	6.901	0.00298	**
Residuals	12	1044	87			
Methanol (72-h)						
Concentration	5	945.4	189.08	15.11	>0.001	***
Residuals	12	150.1	12.51			

B.3 Intrinsic toxicity of TIE manipulation in aqueous sample: Post hoc test for root.

Table B3. 1: Dunnet's multiple comparison (one tailed) for the effect of TIE manipulations (intrinsic toxicity) on the root elongation of *O. sativa* in aqueous samples.

Endpoint: Root length					
EDTA (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
12.5 - 0 >= 0	9.375	3.9737	2.359	0.99975	
25 - 0 >= 0	6.0967	3.9737	1.534	0.99701	
50 - 0 >= 0	8.8182	3.9737	2.219	0.99961	
100 - 0 >= 0	-0.0099	3.9737	-0.002	0.8565	
200 - 0 >= 0	-7.9717	3.9737	-2.006	0.12196	
400 - 0 >= 0	-17.4329	3.9737	-4.387	0.00155	**
EDTA (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	
12.5 - 0 >= 0	2.9284	1.9381	1.511	0.997	
25 - 0 >= 0	2.6954	1.9381	1.391	0.996	
50 - 0 >= 0	2.1097	1.9381	1.089	0.989	
100 - 0 >= 0	-0.2019	1.9381	-0.104	0.827	
200 - 0 >= 0	-3.6903	1.9381	-1.904	0.143	
400 - 0 >= 0	-9.9409	1.9381	-5.129	<0.001	***
STS (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
281.25 - 0 >= 0	8.102	3.776	2.146	0.99952	
562.5 - 0 >= 0	8.473	3.776	2.244	0.99964	
1125 - 0 >= 0	12.325	3.776	3.264	0.99998	
2250 - 0 >= 0	4.427	3.776	1.172	0.99159	
4500 - 0 >= 0	-6.026	3.776	-1.596	0.22504	
9000 - 0 >= 0	-15.51	3.776	-4.107	0.00257	**

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Table B3. 1 – *Continued from previous page*

Endpoint: Root length

STS (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
281.25 - 0 <= 0	8.102	3.776	2.146	0.0972	
562.5 - 0 <= 0	8.473	3.776	2.244	0.0824	
1125 - 0 <= 0	12.325	3.776	3.264	0.0128	*
2250 - 0 <= 0	4.427	3.776	1.172	0.3817	
4500 - 0 <= 0	-6.026	3.776	-1.596	0.9975	
9000 - 0 <= 0	-15.51	3.776	-4.107	1	
STS (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
281.25 - 0 >= 0	1.4876	1.3987	1.064	0.9886	
562.5 - 0 >= 0	2.6244	1.3987	1.876	0.9989	
1125 - 0 >= 0	4.1013	1.3987	2.932	1	
2250 - 0 >= 0	0.6611	1.3987	0.473	0.9477	
4500 - 0 >= 0	-3.2422	1.3987	-2.318	0.0727	.
9000 - 0 >= 0	-7.2035	1.3987	-5.15	<0.001	***

Table B3. 2: Dunnet's multiple comparison (one tailed) for the effect of TIE manipulations (intrinsic toxicity) on the root elongation of *O. sativa* in aqueous samples.

Endpoint: Root length					
STS(72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*))**	**Sig.**
281.25 - 0 <= 0	1.4876	1.3987	1.064	0.4288	
562.5 - 0 <= 0	2.6244	1.3987	1.876	0.1494	
1125 - 0 <= 0	4.1013	1.3987	2.932	0.0242	*
2250 - 0 <= 0	0.6611	1.3987	0.473	0.6932	
4500 - 0 <= 0	-3.2422	1.3987	-2.318	0.9997	
9000 - 0 <= 0	-7.2035	1.3987	-5.15	1	
Methanol (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.389 - 0 >= 0	2.5606	2.8881	0.887	0.97549	
0.779 - 0 >= 0	0.6932	2.8881	0.24	0.89379	
1.559 - 0 >= 0	-10.7519	2.8881	-3.723	0.0058	**
3.118 - 0 >= 0	-13.185	2.8881	-4.565	0.00127	**
6.237 - 0 >= 0	-15.3192	2.8881	-5.304	< 0.001	***

B.4 Intrinsic toxicity of TIE manipulation in aqueous sample: ANOVA summary for shoot.

Table B4. 1: ANOVA for the effect of TIE manipulations (intrinsic toxicity) on the shoot elongation of *O. sativa* in aqueous samples.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
EDTA (96 –hr)						
Concentration	6	30	5	1.053	0.434	
EDTA (72-h)						
Concentration	6	2.105	0.3508	0.198	0.972	
Residuals	14	24.793	1.7709			
STS (96-h)						
Concentration	6	146.3	24.382	9.456	0.000293	***
Residuals	14	36.1	2.579			
STS (72-h)						
Concentration	6	5.598	0.9329	1.687	0.197	
Residuals	14	7.743	0.5531			
Methanol (96-h)						
Concentration	5	1129.5	225.89	15.08	<0.001	***
Residuals	12	179.8	14.98			
Methanol (72-h)						
Concentration	5	255.51	51.1	23.19	<0.001	***
Residuals	12	26.45	2.2			

B.5 Intrinsic toxicity of TIE manipulation in aqueous sample: Post hoc test for shoot.

Table B5. 1: Dunnet's multiple comparison (one tailed) for the effect of TIE manipulations (intrinsic toxicity) on the shoot elongation of *O. sativa* in aqueous samples.

Endpoint: Shoot length					
STS (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
281.25 - 0 >= 0	0.2532	1.3111	0.193	0.9026	
562.5 - 0 >= 0	0.6646	1.3111	0.507	0.9517	
1125 - 0 >= 0	0.7918	1.3111	0.604	0.9619	
2250 - 0 >= 0	-1.9767	1.3111	-1.508	0.2533	
4500 - 0 >= 0	-3.4339	1.3111	-2.619	0.0428	*
9000 - 0 >= 0	-6.9349	1.3111	-5.289	<0.001	***
Methanol (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.389 - 0 >= 0	-2.041	3.16	-0.646	0.58435	
0.779 - 0 >= 0	-3.819	3.16	-1.208	0.33927	
1.559 - 0 >= 0	-12.544	3.16	-3.969	0.00386	**
3.118 - 0 >= 0	-16.451	3.16	-5.206	< 0.001	***
6.237 - 0 >= 0	-21.368	3.16	-6.761	< 0.001	***
Methanol (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.389 - 0 >= 0	-2.297	1.212	-1.895	0.1336	
0.779 - 0 >= 0	-2.658	1.212	-2.193	0.0837	.
1.559 - 0 >= 0	-5.757	1.212	-4.75	<0.001	***
3.118 - 0 >= 0	-9.201	1.212	-7.59	<0.001	***
6.237 - 0 >= 0	-10.402	1.212	-8.581	<0.001	***

B.6 Intrinsic toxicity of TIE manipulation in aqueous sample: ANOVA summary for seedling.

Table B6. 1: ANOVA for the effect of TIE manipulations (intrinsic toxicity) on the seedling length of *O. sativa* in aqueous samples.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
EDTA (96 –hr)						
Cencentration	6	1824.4	304.07	8.743	<0.001	***
Residuals	14	486.9	34.78			
EDTA (72-h)						
Cencentration	6	347.8	57.97	6.928	0.00141	**
Residuals	14	117.1	8.37			
STS (96-h)						
Cencentration	6	2773.3	462.2	14.25	<0.001	***
Residuals	14	454.1	32.4			
STS (72-h)						
Cencentration	6	337.8	56.29	19.71	<0.001	***
Residuals	14	40	2.86			
Methanol (96-h)						
Cencentration	5	7606	1521.2	9.852	<0.001	***
Residuals	12	1853	154.4			
Methanol (72-h)						
Cencentration	5	2118.5	423.7	19.42	<0.001	***
Residuals	12	261.8	21.8			

B.7 Intrinsic toxicity of TIE manipulation in aqueous sample: Post hoc test for seedling.

Table B7. 1: Dunnet's multiple comparison (one tailed) for the effect of TIE manipulations (intrinsic toxicity) on the seedling length of *O. sativa* in aqueous samples.

Endpoint: Seedling length					
EDTA (96–hr)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
12.5 - 0 >= 0	6.7461	4.815	1.401	0.99564	
25 - 0 >= 0	5.3512	4.815	1.111	0.99003	
50 - 0 >= 0	6.9167	4.815	1.436	0.99606	
100 - 0 >= 0	-0.5828	4.815	-0.121	0.82212	
200 - 0 >= 0	-8.2713	4.815	-1.718	0.18928	
400 - 0 >= 0	-20.8239	4.815	-4.325	0.00161	**
EDTA (72–hr)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
12.5 - 0 >= 0	2.4272	2.3618	1.028	0.98742	
25 - 0 >= 0	2.987	2.3618	1.265	0.99353	
50 - 0 >= 0	2.2377	2.3618	0.947	0.98437	
100 - 0 >= 0	0.1611	2.3618	0.068	0.87465	
200 - 0 >= 0	-3.4723	2.3618	-1.47	0.26624	

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Table B7. 1 – *Continued from previous page*

Endpoint: Seedling length					
400 - 0 >= 0	-9.3611	2.3618	-3.963	0.00348	**
STS (96 –hr)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
281.25 - 0 >= 0	8.356	4.65	1.797	0.999	
562.5 - 0 >= 0	9.137	4.65	1.965	0.999	
1125 - 0 >= 0	13.117	4.65	2.821	1	
2250 - 0 >= 0	2.45	4.65	0.527	0.954	
4500 - 0 >= 0	-9.46	4.65	-2.034	0.117	
9000 - 0 >= 0	-22.445	4.65	-4.827	<0.001	
STS (96 –hr)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
281.25 - 0 <= 0	8.356	4.65	1.797	0.1686	
562.5 - 0 <= 0	9.137	4.65	1.965	0.1301	
1125 - 0 <= 0	13.117	4.65	2.821	0.0296	*
2250 - 0 <= 0	2.45	4.65	0.527	0.6704	
4500 - 0 <= 0	-9.46	4.65	-2.034	0.9993	
9000 - 0 <= 0	-22.445	4.65	-4.827	1	

Table B7. 2: Dunnet's multiple comparison (one tailed) for the effect of TIE manipulations (intrinsic toxicity) on the seedling length of *O. sativa* in aqueous samples.

Endpoint: Seedling length					
STS (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
281.25 - 0 >= 0	1.7562	1.3799	1.273	0.9937	
562.5 - 0 >= 0	2.5982	1.3799	1.883	0.9989	
1125 - 0 >= 0	4.679	1.3799	3.391	1	
2250 - 0 >= 0	0.5055	1.3799	0.366	0.9331	
4500 - 0 >= 0	-4.2539	1.3799	-3.083	0.0183	*
9000 - 0 >= 0	-7.9709	1.3799	-5.776	<0.001	***
STS (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
281.25 - 0 <= 0	1.7562	1.3799	1.273	0.3403	
562.5 - 0 <= 0	2.5982	1.3799	1.883	0.148	
1125 - 0 <= 0	4.679	1.3799	3.391	0.0101	*
2250 - 0 <= 0	0.5055	1.3799	0.366	0.736	
4500 - 0 <= 0	-4.2539	1.3799	-3.083	1	
9000 - 0 <= 0	-7.9709	1.3799	-5.776	1	
Methanol (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.389 - 0 >= 0	8.7652	10.1458	0.864	0.9741	
0.779 - 0 >= 0	0.2765	10.1458	0.027	0.8412	
1.559 - 0 >= 0	-22.7688	10.1458	-2.244	0.0769	.
3.118 - 0 >= 0	-33.4802	10.1458	-3.3	0.0123	*
6.237 - 0 >= 0	-48.4	10.1458	-4.77	<0.001	***
Methanol (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.389 - 0 >= 0	0.2633	3.8135	0.069	0.85272	
0.779 - 0 >= 0	-1.9649	3.8135	-0.515	0.64209	
1.559 - 0 >= 0	-16.509	3.8135	-4.329	0.00189	**
3.118 - 0 >= 0	-22.3856	3.8135	-5.87	<0.001	***
6.237 - 0 >= 0	-25.7209	3.8135	-6.745	<0.001	***

B.8 Intrinsic toxicity of TIE manipulation in aqueous sample: ANOVA summary for seed germination.

Table B8. 1: ANOVA for the effect of TIE manipulations (intrinsic toxicity) on the seed germination of *O. sativa* in aqueous samples.

EDTA (96 –hr)						
	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Cencentration	6	5.143	0.8571	1	0.463	
Residuals	14	12	0.8571			
EDTA (72-h)						
Cencentration	6	6.476	1.0794	1.619	0.214	
Residuals	14	9.333	0.6667			
STS (96-h)						
Cencentration	6	2.476	0.4127	1.238	0.345	
Residuals	14	4.667	0.3333			
STS (72-h)						
Cencentration	6	13.81	2.302	0.948	0.493	
Residuals	14	34	2.429			
Methanol (96-h)						
Cencentration	6	352.6	58.76	22.85	<0.001	***
Residuals	14	36	2.57			
Methanol (72-h)						
Cencentration	6	303.9	50.65	13.3	<0.001	***
Residuals	14	53.33	3.81			

B.9 Intrinsic toxicity of TIE manipulation in aqueous sample: Post hoc test for seed germination.

Table B9. 1: Dunnet's multiple comparison (one tailed) for the effect of TIE manipulations (intrinsic toxicity) on the seed germination of *O. sativa* in aqueous samples.

Endpoint: Seed germination					
Methanol (96-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.389 - 0 >= 0	-0.3333	1.3093	-0.255	0.7776	
0.779 - 0 >= 0	-1	1.3093	-0.764	0.5648	
1.559 - 0 >= 0	-1.3333	1.3093	-1.018	0.4488	
3.118 - 0 >= 0	-4.3333	1.3093	-3.31	0.0118	*
6.237 - 0 >= 0	-10	1.3093	-7.638	<0.001	***
12.475 - 0 >= 0	-10	1.3093	-7.638	<0.001	***
Methanol (72-h)	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.389 - 0 >= 0	-0.6667	1.5936	-0.418	0.7155	
0.779 - 0 >= 0	-0.6667	1.5936	-0.418	0.7155	
1.559 - 0 >= 0	-5	1.5936	-3.137	0.0166	*
3.118 - 0 >= 0	-6.6667	1.5936	-4.183	0.0023	**
6.237 - 0 >= 0	-9.3333	1.5936	-5.857	<0.001	***
12.475 - 0 >= 0	-9.3333	1.5936	-5.857	<0.001	***

Appendix C

Chapter 4

C.1 Two-way ANOVA for sediment monitoring study: Physicochemical Variables.

Table C1. 1: Two-way ANOVA output for the physicochemical variables of sediment collected from the River Periyar.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
			Temperature			
sites	2	0.52	0.26	0.875	0.43386	
season	2	65.41	32.7	110.375	7.87E-11	***
sites:season	4	6.81	1.7	5.75	0.00366	**
Residuals	18	5.33	0.3			
			pH			
site	2	6.838	3.419	14.702	0.000164	***
season	2	3.181	1.591	6.84	0.006172	**
site:season	4	2.548	0.637	2.739	0.061032	.
Residuals	18	4.186	0.233			
			EC			
sites	2	3.656	1.8278	37.22	4.02E-07	***
season	2	5.46	2.73	55.59	1.98E-08	***
sites:season	4	3.245	0.8112	16.52	7.63E-06	***
Residuals	18	0.884	0.0491			
			ORP			
sites	2	994230	497115	5.627	0.012637	*
season	2	2122541	1061270	12.014	0.000485	***
sites:season	4	787570	196893	2.229	0.106522	
Residuals	18	1590067	88337			

Table C1. 2: Two-way ANOVA output for the physicochemical variables of sediment collected from the River Periyar.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
			TAN			
sites	2	117.31	58.65	9.979	0.00121	**
season	2	1.23	0.61	0.105	0.90124	
sites:season	4	8.45	2.11	0.359	0.83411	
Residuals	18	105.8	5.88			
			TOC			
sites	2	10.796	5.398	7.111	0.0053	**
season	2	2.867	1.433	1.888	0.1801	
sites:season	4	9.805	2.451	3.229	0.0366	*
Residuals	18	13.663	0.759			
			P			
site	2	75.1	37.53	0.328	0.725	
season	2	14.4	7.19	0.063	0.939	
site:season	4	309.9	77.47	0.677	0.617	
Residuals	18	2060.3	114.46			
			K			
sites	2	24368	12184	2.125	0.148	
season	2	19048	9524	1.661	0.218	
sites:season	4	20099	5025	0.877	0.497	
Residuals	18	103182	5732			

Table C1. 3: Two-way ANOVA output for the physicochemical variables of sediment collected from the River Periyar.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
			TS			
site	2	1814.6	907.3	31.239	1.40E-06	***
season	2	65.4	32.7	1.126	0.3463	
site:season	4	292.9	73.2	2.521	0.0772	.
Residuals	18	522.8	29			
			Sand			
site	2	488	244.2	0.432	0.656	
season	2	518	259	0.458	0.64	
site:season	4	758	189.4	0.335	0.851	
Residuals	18	10175	565.3	0.432	0.656	
			Silt			
site	2	243	121.29	0.562	0.58	
season	2	320	160.23	0.742	0.49	
site:season	4	155	38.87	0.18	0.946	
Residuals	18	3886	215.87	0.562	0.58	
			Clay			
site	2	220.4	110.18	0.715	0.503	
season	2	44.3	22.13	0.144	0.867	
site:season	4	349.8	87.44	0.567	0.69	
Residuals	18	2775.5	154.19			

C.2 Post hoc comparison for sediment monitoring study: Physicochemical Variables.

Table C2. 1: Post hoc comparison (Tukey's HSD test) of temperature of sediment collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014). The numbers 1, 2, and 3 denote stations 1, 2, and 3, respectively; "mon", "post", and "pre" denote monsoon, post-monsoon, and pre-monsoon seasons, respectively.

Temperature

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Season = mon					
s1 - s2	6.70E-01	4.40E-01	1.80E+01	1.50E+00	0.3144
s1 - s3	-1.00E+00	4.40E-01	1.80E+01	-2.30E+00	0.0896
s2 - s3	-1.70E+00	4.40E-01	1.80E+01	-3.80E+00	0.004
Season = post					
s1 - s2	1.10E-16	4.40E-01	1.80E+01	0.00E+00	1
s1 - s3	-3.30E-01	4.40E-01	1.80E+01	-7.50E-01	0.7375
s2 - s3	-3.30E-01	4.40E-01	1.80E+01	-7.50E-01	0.7375
Season = pre					
s1 - s2	-1.00E+00	4.40E-01	1.80E+01	-2.30E+00	0.0896
s1 - s3	3.30E-01	4.40E-01	1.80E+01	7.50E-01	0.7375
s2 - s3	1.30E+00	4.40E-01	1.80E+01	3.00E+00	0.02

Table C2. 2: Post hoc comparison (Tukey's HSDT test) of pH of sediment collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014). The numbers 1, 2, and 3 denote stations 1, 2, and 3, respectively; "mon", "post", and "pre" denote monsoon, post-monsoon, and pre-monsoon seasons, respectively.

pH

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between stations					
s1 - s2	-0.769	0.227	18	-3.382	0.0089
s1 - s3	-1.219	0.227	18	-5.362	0.0001
s2 - s3	-0.45	0.227	18	-1.979	0.146
Between seasons					
mon - post	0.84	0.227	18	3.695	0.0045
mon - pre	0.452	0.227	18	1.989	0.1436
post - pre	-0.388	0.227	18	-1.706	0.2302

Table C2. 3: Post hoc comparison (Tukey's HSD test) of electrical conductivity (EC) of sediment collected from three stations of the River Periyar (the values averaged across seasons). The numbers 1, 2, and 3 denote stations 1, 2, and 3, respectively. "mon", "post", and "pre" denote monsoon, post-monsoon, and pre-monsoon seasons, respectively.

	Contrast	Estimate	SE	df	*t*-ratio	*p*-value
EC	**Season = mon**					
	s1 - s2	-0.31	0.181	18	-1.713	0.2275
	s1 - s3	-0.26	0.181	18	-1.437	0.3438
	s2 - s3	0.05	0.181	18	0.276	0.9589
	Season = post					
	s1 - s2	-1.313	0.181	18	-7.258	<.0001
	s1 - s3	-2.067	0.181	18	-11.422	<.0001
	s2 - s3	-0.753	0.181	18	-4.163	0.0016
	Season = pre					
	s1 - s2	-0.303	0.181	18	-1.676	0.2413
	s1 - s3	-0.28	0.181	18	-1.546	0.294
	s2 - s3	0.024	0.181	18	0.13	0.9907

Table C2. 4: Post hoc comparison (Tukey's HSD test) of ORP of sediment collected from three stations of the River Periyar (the values averaged across seasons). The numbers 1, 2, and 3 denote stations 1, 2, and 3, respectively.

	Contrast	Estimate	SE	df	*t*-ratio	*p*-value
ORP	**Between stations**					
	s1 - s2	288.89	140.11	18	2.062	0.1263
	s1 - s3	465.56	140.11	18	3.323	0.0101
	s2 - s3	176.67	140.11	18	1.261	0.4344

Table C2. 5: Post hoc comparison (Tukey's HSD test) of total ammonia nitrogen (TAN) of sediment collected from three stations of the River Periyar (the values averaged across seasons). The numbers 1, 2, and 3 denote stations 1, 2, and 3, respectively.

	Contrast	Estimate	SE	df	*t*-ratio	*p*-value
TAN	**Between stations**					
	s1 - s2	3.584	1.143	18	3.136	0.015
	s1 - s3	-1.358	1.143	18	-1.188	0.4752
	s2 - s3	-4.941	1.143	18	-4.323	0.0011

Table C2. 6: Post hoc comparison (Tukey's HSD test) of total organic carbon (TOC) of sediment collected from three stations of the River Periyar (the values averaged across seasons). The numbers 1, 2, and 3 denote stations 1, 2, and 3, respectively.

	Contrast	Estimate	SE	df	*t*-ratio	*p*-value
TOC	**Season = mon**					
	s1 - s2	-0.893	0.711	18	-1.256	0.4372
	s1 - s3	-1.977	0.711	18	-2.779	0.0317
	s2 - s3	-1.083	0.711	18	-1.523	0.3041
	Season = post					
	s1 - s2	2.627	0.711	18	3.692	0.0045
	s1 - s3	0.29	0.711	18	0.408	0.9129
	s2 - s3	-2.337	0.711	18	-3.285	0.0109
	Season = pre					
	s1 - s2	0.74	0.711	18	1.04	0.5619
	s1 - s3	-0.483	0.711	18	-0.679	0.7782
	s2 - s3	-1.223	0.711	18	-1.72	0.2252

Table C2. 7: Post hoc comparison (Tukey's HSD test) of total solids (TS) of sediment collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014). The numbers 1, 2, and 3 denote stations 1, 2, and 3, respectively.

	Contrast	**Estimate**	**SE**	**df**	***t*-ratio**	***p*-value**
TS	**Between stations**					
	s1 - s2	-0.701	2.541	18	-0.276	0.9589
	s1 - s3	17.029	2.541	18	6.703	<.0001
	s2 - s3	17.731	2.541	18	6.979	<.0001

C.3 Two-way ANOVA for sediment monitoring study: Biological Variables.

Table C3. 1: Two-way ANOVA for morphometric variables (4-day) of *O. sativa* var. Jyothi grown in sediments collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014).

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Root length (4-days)						
sites	2	14911	7456	25.558	5.51E-06	***
season	2	675	337	1.156	0.337	
sites:season	4	591	148	0.506	0.732	
Residuals	18	5251	292			
Shoot length (4-days)						
sites	2	3334	1667	25.89	5.06E-06	***
season	2	540	269.9	4.191	0.032	*
sites:season	4	11	2.8	0.043	0.996	
Residuals	18	1159	64.4			
Seedling length (4-days)						
sites	2	32305	16153	26.559	4.26E-06	***
season	2	2420	1210	1.989	0.166	
sites:season	4	702	175	0.288	0.882	
Residuals	18	10947	608			

Table C3. 2: Two-way ANOVA for morphometric variables (7-day) of *O. sativa* var. Jyothi grown in sediments collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014).

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Root length (7-days)						
sites	2	31322	15661	20.993	1.97E-05	***
season	2	3175	1588	2.128	0.148	
sites:season	4	5296	1324	1.775	0.178	
Residuals	18	13428	746			
Shoot length (7-days)						
sites	2	6524	3262	13.285	0.000286	***
season	2	3053	1527	6.218	0.008852	**
sites:season	4	1797	449	1.829	0.16714	
Residuals	18	4420	246			
Seedling length (7-days)						
sites	2	64810	32405	18.503	4.30E-05	***
season	2	11096	5548	3.168	0.0663	.
sites:season	4	10032	2508	1.432	0.2639	
Residuals	18	31525	1751			

Table C3. 3: One-way ANOVA for morphometric variables (4-day) of salt tolerant variety (Vyttila-6) of *O. sativa* grown in sediments collected from three stations of the River Periyar during post-monsoon of 2013.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Root length (4-days)						
sites	2	6401.2	3200.6	54.532	0.000142	***
Residuals	6	352.1	58.7			
Shoot length (4-days)						
sites	2	1402.11	701.05	115.27	1.63E-05	***
Residuals	6	36.49	6.08			
Seedling length (4-days)						
sites	2	6401.2	3200.6	54.532	0.000142	***
Residuals	6	352.1	58.7			

Table C3. 4: One-way ANOVA for morphometric variables (7-day) of salt tolerant variety (Vyttila-6) of *O. sativa* grown in sediments collected from three stations of the River Periyar during post-monsoon of 2013.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Root length (7-days)						
sites	2	17221.5	8610.8	20.298	0.002135	**
Residuals	6	2545.3	424.2			
Shoot length (7-days)						
sites	2	3009.91	1504.96	11.836	0.008268	**
Residuals	6	762.88	127.15			
Seedling length (7-days)						
sites	2	34249	17124.7	22.015	0.001725	**
Residuals	6	4667	777.9			

C.4 Post hoc comparison for the sediment monitoring study: Biological variables.

Table C4. 1: Post hoc comparison (Tukey's HSD test) for root length of *O. sativa* var. Jyothi grown (4-days) in sediments collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Root (4-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	44.529	8.051	18	5.531	0.0001
s1 - s3	53.857	8.051	18	6.689	<.0001
s2 - s3	9.329	8.051	18	1.159	0.492

Table C4. 2: Post hoc comparison (Tukey's HSD test) for shoot length of *O. sativa* var. Jyothi grown (4-days) in sediments collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014); "mon", "post", and "pre" denote monsoon, post-monsoon, and pre-monsoon, respectively; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Shoot (4-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	22.321	3.783	18	5.901	<.0001
s1 - s3	24.651	3.783	18	6.517	<.0001
s2 - s3	2.331	3.783	18	0.616	0.8132
Between seasons					
mon - post	9.293	3.783	18	2.457	0.0603
mon - pre	-0.372	3.783	18	-0.098	0.9947
post - pre	-9.665	3.783	18	-2.555	0.0497

Table C4. 3: Post hoc comparison (Tukey's HSD test) for seedling length of *O. sativa* var. Jyothi grown (4-days) in sediments collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014); s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Seedling (4-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	66.849	11.625	18	5.75	0.0001
s1 - s3	78.509	11.625	18	6.753	<.0001
s2 - s3	11.659	11.625	18	1.003	0.5845

Table C4. 4: Post hoc comparison (Tukey's HSD test) for root length of *O. sativa* var. Jyothi grown (7-days) in sediments collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014); s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Root (7-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	70.73	12.876	18	5.493	0.0001
s1 - s3	73.684	12.876	18	5.723	0.0001
s2 - s3	2.954	12.876	18	0.229	0.9715

Table C4. 5: Post hoc comparison (Tukey's HSD test) for shoot length of *O. sativa* var. Jyothi grown (7-days) in sediments collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014); "mon", "post", and "pre" denote monsoon, post-monsoon, and pre-monsoon, respectively; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

	Contrast	**Estimate**	**SE**	**df**	***t*-ratio**	***p*-value**
Shoot (7-day)	**Between sites**					
	s1 - s2	34.222	7.387	18	4.633	0.0006
	s1 - s3	31.566	7.387	18	4.273	0.0013
	s2 - s3	-2.656	7.387	18	-0.36	0.9315
	Between seasons					
	mon - post	19.472	7.387	18	2.636	0.0423
	mon - pre	-5.248	7.387	18	-0.71	0.7605
	post - pre	-24.72	7.387	18	-3.347	0.0096

Table C4. 6: Post hoc comparison (Tukey's HSD test) for seedling length of *O. sativa* var. Jyothi grown (7-days) in sediments collected from three stations of the River Periyar during three seasons (from monsoon of 2013 to pre monsoon of 2014); s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Seedling (7-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	104.952	19.728	18	5.32	0.0001
s1 - s3	102.88	19.728	18	5.215	0.0002
s2 - s3	-2.072	19.728	18	-0.105	0.9939

Table C4. 7: Post hoc comparison (Tukey's HSD test) for root length of salt tolerant variety (Vyttila-6) *O. sativa* grown (4-days) in sediments collected from three stations of the River Periyar during post-monsoon of 2013; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Root (4-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	41.433	6.255	6	6.624	0.0014
s1 - s3	64.455	6.255	6	10.304	0.0001
s2 - s3	23.022	6.255	6	3.681	0.0241

Table C4. 8: Post hoc comparison (Tukey's HSD test) for shoot length of salt tolerant variety (Vyttila-6) *O. sativa* grown (4-days) in sediments collected from three stations of the River Periyar during post-monsoon of 2013; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Shoot (4-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	22.7584	2.0136	6	11.302	0.0001
s1 - s3	29.0596	2.0136	6	14.431	<.0001
s2 - s3	6.3011	2.0136	6	3.129	0.0464

Table C4. 9: Post hoc comparison (Tukey's HSD test) for seedling length of salt tolerant variety (Vyttila-6) *O. sativa* grown (4-days) in sediments collected from three stations of the River Periyar during during post-monsoon of 2013; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Seedling (4-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	64.191	7.776	6	8.255	0.0004
s1 - s3	93.515	7.776	6	12.026	<.0001
s2 - s3	29.324	7.776	6	3.771	0.0217

Table C4. 10: Post hoc comparison (Tukey's HSD test) for root length of salt tolerant variety (Vyttila-6) *O. sativa* grown (7-days) in sediments collected from three stations of the River Periyar during during post-monsoon of 2013; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Root (7-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	76.021	16.817	6	4.52	0.0095
s1 - s3	103.405	16.817	6	6.149	0.0021
s2 - s3	27.384	16.817	6	1.628	0.3054

Table C4. 11: Post hoc comparison (Tukey's HSD test) for shoot length of salt tolerant variety (Vyttila-6) *O. sativa* grown (7-days) in sediments collected from three stations of the River Periyar during post-monsoon of 2013; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Shoot (7-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	23.722	9.207	6	2.577	0.0926
s1 - s3	44.769	9.207	6	4.863	0.0067
s2 - s3	21.047	9.207	6	2.286	0.1341

Table C4. 12: Post hoc comparison (Tukey's HSD test) for seedling length of salt tolerant variety (Vyttila-6) *O. sativa* grown (7-days) in sediments collected from three stations of the River Periyar during post-monsoon of 2013; s1, s2, and s3 denote stations 1, 2, and 3, respectively.

Seedling (7-days)

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Between sites					
s1 - s2	99.742	22.772	6	4.38	0.0111
s1 - s3	148.173	22.772	6	6.507	0.0015
s2 - s3	48.431	22.772	6	2.127	0.1643

Appendix D

Chapter 5

D.1 ANOVA output for intrinsic toxicity in Sediment TIE

Table D1. 1: One-way ANOVA output for the impact of cation exchange resin (CER) treatment in OECD sediment on various morphological responses *O. sativa*.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Root						
Concentration	4	7981	1995.2	49.62	<0.001	***
Residuals	10	402	40.2			
Shoot						
Concentration	4	141.9	35.49	2.567	0.103	
Residuals	10	138.2	13.82			
Seedling						
Concentration	4	9506	2376.5	28.07	<0.001	***
Residuals	10	847	84.7			

Table D1. 2: One-way ANOVA output for the impact of charcoal treatment in OECD sediment on various morphological responses *O. sativa*.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Root						
Concentration	3	3007	1002.4	7.14	0.0119	*
Residuals	8	1123	140.4			
Shoot						
Concentration	3	123.6	41.19	1.193	0.372	
Residuals	8	276.2	34.53			
Seedling						
Concentration	3	3986	1328.8	5.645	0.0225	*
Residuals	8	1883	235.4			

Table D1. 3: One-way ANOVA output for the impact of sulfide (Na_2S) treatment in OECD sediment on various morphological responses *O. sativa*.

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Root						
Concentration	3	7447	2482.2	55.55	<0.001	***
Residuals	8	357	44.7			
Shoot						
Concentration	3	1587.7	529.2	47.69	<0.001	***
Residuals	8	88.8	11.1			
Seedling						
Concentration	3	15851	5284	54.91	<0.001	***
Residuals	8	770	96			

Table D1. 4: One-way ANOVA output for the impact of zeolite treatment in OECD sediment on various morphological responses *O. sativa*

	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Root						
Concentration	4	7978	1994.4	36.55	<0.001	***
Residuals	10	546	54.6			
Shoot						
Concentration	4	60.54	15.13	1.282	0.34	
Residuals	10	118.01	11.8			
Seedling						
Concentration	4	8508	2127	20.4	<0.001	***
Residuals	10	1043	104.3			

D.2 Post hoc Tests for intrinsic toxicity in Sediment TIE.

Table D2. 1: post hoc comparison (Tukey's HSD) for the impact of cation exchange resin (CER) treatment in OECD sediment on various morphological responses *O. sativa*.

Root					
Concentration	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
0.625 - 0 >= 0	0.2856	5.1775	0.055	0.521	
2.5 - 0 >= 0	-45.7771	5.1775	-8.842	<0.001	***
5 - 0 >= 0	-44.0717	5.1775	-8.512	<0.001	***
10 - 0 >= 0	-50.4017	5.1775	-9.735	<0.001	***
Shoot					
0.625 - 0 >= 0	4.67063	3.03571	1.539	1	
2.5 - 0 >= 0	0.04593	3.03571	0.015	1	
5 - 0 >= 0	0.78423	3.03571	0.258	1	
10 - 0 >= 0	-4.99373	3.03571	-1.645	0.262	
Seedling					
0.625 - 0 >= 0	4.956	7.512	0.66	0.7378	
2.5 - 0 >= 0	-45.731	7.512	-6.087	<0.001	***
5 - 0 >= 0	-43.287	7.512	-5.762	<0.001	***
10 - 0 >= 0	-55.395	7.512	-7.374	<0.001	***

Table D2. 2: Post hoc comparison (Tukey's HSD) for the impact of charcoal treatment in OECD sediment on various morphological responses *O. sativa*.

Root					
Concentration	**Estimate**	**Std. Error**	***t*-value**	**Pr(>t)**	**Sig.**
1 - 0 <= 0	25.762	9.674	2.663	0.01434	*
2 - 0 <= 0	35.094	9.674	3.628	0.00671	**
5 - 0 <= 0	41.627	9.674	4.303	0.00391	**
Shoot					
1 - 0 <= 0	2.442	4.798	0.509	0.464	
2 - 0 <= 0	8.791	4.798	1.832	0.156	
5 - 0 <= 0	3.686	4.798	0.768	0.464	
Seedling					
1 - 0 <= 0	28.2	12.53	2.251	0.0272	*
2 - 0 <= 0	43.89	12.53	3.503	0.0102	*
5 - 0 <= 0	45.31	12.53	3.617	0.0102	*

Table D2. 3: Post hoc comparison (Tukey's HSD) for the impact of sulfide (Na_2S) treatment in OECD sediment on various morphological responses *O. sativa*.

Concentrations	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
Root					
5 - 0 >= 0	3.526	5.458	0.646	0.731817	
10 - 0 >= 0	-32.908	5.458	-6.03	<0.001	***
20 - 0 >= 0	-57.046	5.458	-10.452	<0.001	***
Shoot					
5 - 0 >= 0	0.04407	2.72004	0.016	0.50626	
10 - 0 >= 0	-13.0444	2.72004	-4.796	0.00136	**
20 - 0 >= 0	-27.8678	2.72004	-10.245	<0.001	***
Seedling					
5 - 0 >= 0	3.57	8.009	0.446	0.666187	
10 - 0 >= 0	-45.953	8.009	-5.737	<0.001	***
20 - 0 >= 0	-84.914	8.009	-10.602	<0.001	***

Table D2. 4: Post hoc comparison (Tukey's HSD) for the impact of zeolite treatment in OECD sediment on various morphological responses *O. sativa*.

Concentrations	**Estimate**	**Std. Error**	***t*-value**	**Pr(>*t*)**	**Sig.**
Root					
0.625 - 0 >= 0	-0.5799	6.0312	-0.096	0.463	
2.5 - 0 >= 0	-41.1285	6.0312	-6.819	<0.001	***
5 - 0 >= 0	-49.5002	6.0312	-8.207	<0.001	***
10 - 0 >= 0	-50.1143	6.0312	-8.309	<0.001	***
Shoot					
0.625 - 0 >= 0	2.3509	2.8049	0.838	1	
2.5 - 0 >= 0	3.3569	2.8049	1.197	1	
5 - 0 >= 0	-2.2598	2.8049	-0.806	0.878	
10 - 0 >= 0	-0.3874	2.8049	-0.138	1	
Seedling					
0.625 - 0 >= 0	1.771	8.338	0.212	0.5820	
2.5 - 0 >= 0	-37.772	8.338	-4.53	0.0011	**
5 - 0 >= 0	-51.76	8.338	-6.208	0.0002	***
10 - 0 >= 0	-50.502	8.338	-6.057	0.0002	***

D.3 Sediment TIE: Kruskal-Wallis rank sum test and One-way ANOVA.

Table D3. 1: Kruskal-Wallis rank sum test for the impact of sediment TIE on root *O. sativa*.

Root
Kruskal-Wallis rank sum test
data: root by treat
Kruskal-Wallis chi-squared = 28.305, df = 6, ***p***-value = 8.232e-05

Table D3. 2: One-way ANOVA output for the impact of sediment TIE on various morphological responses *O. sativa*.

Shoot						
	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Treat	6	3.624	0.604	30.95	<0.001	***
Residuals	28	0.546	0.0195			
Seedling						
Treat	6	1.6073	0.26789	21.15	<0.001	***
Residuals	28	0.3546	0.01267			

Table D3. 3: Dunn's multiple comparison (1964) for the impact of sediment TIE on root length *O. sativa*. *p*-values adjusted with the Benjamini-Hochberg method. Kruskal-Wallis test yielded chi-squared = 28.305, df = 6, and *p*-value = 8.232e-05.

Contrasts	**Z**	***p*-unadj**	***p*-adj**
Baseline - CER	-1.450	1.5E-01	0.2374
Baseline - Charcoal	-2.623	8.7E-03	0.0305
CER - Charcoal	-1.173	2.4E-01	0.3162
Baseline - Control	-4.166	3.1E-05	<0.001
CER – Control	-2.716	6.6E-03	0.0278
Charcoal - Control	-1.543	1.2E-01	0.2149
Baseline – Dilution blank	-0.247	8.1E-01	0.8452
CER - Dilution blank	1.204	2.3E-01	0.3203
Charcoal - Dilution blank	2.376	1.8E-02	0.0459
Control - Dilution blank	3.919	8.9E-05	0.0009
Baseline - Sulfide	-3.271	1.1E-03	0.0075
CER - Sulfide	-1.821	6.9E-02	0.1602
Charcoal - Sulfide	-0.648	5.2E-01	0.5714
Control - Sulfide	0.895	3.7E-01	0.4326
Dilution blank - Sulfide	-3.024	2.5E-03	0.0131
Baseline - Zeolite	-1.636	1.0E-01	0.1946
CER - Zeolite	-0.185	8.5E-01	0.8531
Charcoal - Zeolite	0.988	3.2E-01	0.3995
Control - Zeolite	2.531	1.1E-02	0.0342
Dilution blank - Zeolite	-1.389	1.7E-01	0.2474
Sulfide - Zeolite	1.636	1.0E-01	0.2140

Table D3. 4: Post hoc test (Tukey's comparisons) for the impact of sediment TIE on shoot length *O. sativa*.

Contrasts	**Estimate**	**SE**	**df**	***t*-ratio**	***p*-value**
Baseline - CER	0.081	0.088	28	0.917	0.9667
Baseline - Charcoal	-0.536	0.088	28	-6.066	<0.001
Baseline - Control	-0.892	0.088	28	-10.1	<0.001
Baseline – Dilution blank	-0.220	0.088	28	-2.485	0.2029
Baseline - Sulfide	-0.370	0.088	28	-4.191	0.0042
Baseline - Zeolite	-0.027	0.088	28	-0.31	0.9999
CER - Charcoal	-0.617	0.088	28	-6.983	<0.001
CER - Control	-0.973	0.088	28	-11.017	<0.001
CER - Dilution blank	-0.301	0.088	28	-3.402	0.0295
CER - Sulfide	-0.451	0.088	28	-5.108	0.0004
CER - Zeolite	-0.108	0.088	28	-1.227	0.8775
Charcoal - Control	-0.356	0.088	28	-4.034	0.0062
Charcoal - Dilution blank	0.316	0.088	28	3.581	0.0192
Charcoal - Sulfide	0.166	0.088	28	1.875	0.5119
Charcoal - Zeolite	0.509	0.088	28	5.756	<0.001
Control - Dilution blank	0.673	0.088	28	7.615	<.0001
Control - Sulfide	0.522	0.088	28	5.909	<0.001
Control - Zeolite	0.865	0.088	28	9.79	<0.001
Dilution blank - Sulfide	-0.151	0.088	28	-1.706	0.618
Dilution blank - Zeolite	0.192	0.088	28	2.175	0.34
Sulfide - Zeolite	0.343	0.088	28	3.881	0.0092

Table D3. 5: Post hoc test (Tukey's comparisons) for the impact of sediment TIE on seedling length *O. sativa*.

Contrast	Estimate	SE	df	*t*-ratio	*p*-value
Baseline - CER	-0.190	0.071	28	-2.673	0.1429
Baseline - Charcoal	-0.429	0.071	28	-6.026	<0.001
Baseline - Control	-0.650	0.071	28	-9.129	<0.001
Baseline – Dilution blank	-0.083	0.071	28	-1.168	0.9001
Baseline - Sulfide	-0.469	0.071	28	-6.594	<0.001
Baseline - Zeolite	-0.228	0.071	28	-3.208	0.0461
CER - Charcoal	-0.239	0.071	28	-3.353	0.0331
CER - Control	-0.460	0.071	28	-6.456	<0.001
CER - Dilution blank	0.107	0.071	28	1.505	0.7398
CER - Sulfide	-0.279	0.071	28	-3.921	0.0083
CER - Zeolite	-0.038	0.071	28	-0.536	0.998
Charcoal - Control	-0.221	0.071	28	-3.103	0.0583
Charcoal - Dilution blank	0.346	0.071	28	4.858	<0.001
Charcoal - Sulfide	-0.040	0.071	28	-0.568	0.9972
Charcoal - Zeolite	0.201	0.071	28	2.817	0.1071
Control - Dilution blank	0.567	0.071	28	7.961	<0.001
Control - Sulfide	0.180	0.071	28	2.535	0.1854
Control - Zeolite	0.421	0.071	28	5.92	<.0001
Dilution blank - Sulfide	-0.386	0.071	28	-5.426	<0.001
Dilution blank - Zeolite	-0.145	0.071	28	-2.04	0.4135
Sulfide - Zeolite	0.241	0.071	28	3.386	0.0306

Table D3. 6: One-way ANOVA output for the impact of sediment (sieved) TIE on various morphological responses *O. sativa*.

Root						
	Df	**Sum Sq**	**Mean Sq**	***F*-value**	**Pr(>*F*)**	**Sig.**
Treatment	2	347.2	173.61	9.859	0.00293	**
Residuals	12	211.3	17.61			
Shoot						
Treatment	2	16.5	8.249	2.318	0.141	
Residuals	12	42.71	3.559			
Seedling						
Treatment	2	512	256.01	7.654	0.0072	**
Residuals	12	401.4	33.45			

Table D3. 7: Post hoc test (Tukey's comparisons) for the impact of sediment (sieved) TIE on root length *O. sativa*.

Contrasts	**Estimate**	**SE**	**df**	***t*-ratio**	***p*-value**
Baseline - CER	-10.4577	2.653986	12	-3.94	0.0051
Baseline – Dilution blank	-0.5229	2.653986	12	-0.197	0.9789
CER - Dilution blank	9.93478	2.653986	12	3.743	0.0073

Table D3. 8: Post hoc test (Tukey's comparisons) for the impact of sediment (sieved) TIE on seedling length *O. sativa*.

Contrasts	**Estimate**	**SE**	**df**	***t*-ratio**	***p*-value*p*-value**
Baseline - CER	-12.929	3.658	12	-3.534	0.0106
Baseline - Dilution blank	-1.149	3.658	12	-0.314	0.9473
CER - Dilution blank	11.779	3.658	12	3.22	0.0187

D.4 Photographs of sediment TIE experiment.

Figure D4. 1: *O. sativa* grown in OECD (control) sediment.

Figure D4. 2: *O. sativa* grown in 20% CER in OECD sediment.

Figure D4. 3: *O. sativa* grown in 5% coconut charcoal in OECD sediment.

Figure D4. 4: *O. sativa* grown in grown in 20% zeolite in OECD sediment.

Figure D4. 5: *O. sativa* grown in 20% sodium sulfide in OECD sediment.

Appendix E

List of Publications

E.1 Journal Publications

1. **Ajitha, V., Rajathy, S., Rojith, G., Syamkumar, R., 2015**. Physico-chemical characterization of coir pith black liquor and coir pith effluent. Int. Res. J. Environ. Sci. 4, 46–49.

2. **Syamkumar, R., Rojith, G., Rajathy, S., Bright, S.I.S., 2014**. Phytotoxicity assessment of coir pith effluent generated during lignin recovery process. Res. J. Chem. Sci. 4, 17–21.

3. **Syamkumar, R., Rojith, G., Rajathy, S., Bright, S.I.S., 2014**. Evaluation of phytotoxicity of coir pith black liquor generated by oxidative delignification process to *Oryza sativa* L. BTAIJ 9, 391–396.

E.2 Conferences

1. **Loveson, A., Rajathy, S., Syamkumar, R., 2013**. Aquatic

macrophyte *Spirodella polyrhiza* as a phytoremediation tool in polluted back water wet land sites of Kannamaly, ernakulam district, Kerala, in: Conservation of Wetland Ecosystem in Kerala. Presented at the Swadesi Science Congress, M.G. University, Kottayam, Kerala, pp. 493–500.

2. **Syamkumar, R., Rojith, G., Rajathy, S., Bright Singh, I.S., 2013**. Phytotoxicity assessment of coir pith effluent generated during lignin recovery process, in: ISC-2013. Presented at the International Science Congress, International Science Congress Association, Karunya University, Coimbatore.

3. **Syamkumar, R., Rojith, G., Rajathy, S., Bright Singh, I.S., 2013**. Evaluation of phytotoxicity of coir pith black liquor generated by oxidative delignification process to *Oryza sativa* L., in: ICGeT-2013. Presented at the International Conference on Green Technology, School of Chemical and Biotechnology (SCBT), Shanmugha Arts, Science, Technology and Research Academy, SASTRA University, Tirumalaisamudram.

4. **Syamkumar, R., Rojith, G., Rajathy, S., Rajathy, S., Bright Singh, I.S., 2014**. Effect of lignin recovery from coir pith black liquor on phytotoxicity to *Oryza sativa* L, in: ICEE-2014. Presented at the International conference on Environment and Energy, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad.

E.3 Awards

Excellent paper award:

1. **Anand, M., Bright Singh, I.S., Chandini, P.K., Nair, H.M.V., Mithun, A.M., Samitha, K.A., Syamkumar, R., Unnikkuttan, B., 2016**. In-vessel composting of food waste: a novel approach, in: The Sixth International Conference on Solid Waste Management. Presented at the IconSWM-2016, Jadavpur University, Kolkata, W. Bengal, India.

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Cedergreen, Nina, Jens Streibig, Per Kudsk, Solvejg Mathiassen, and Stephen Duke (2007). "The occurrence of hormesis in plants and algae". In: *Dose-Response* 5.2, pp. 150–162. ISSN: 1559-3258. DOI: 10.2203/dose-response.06-008.Cedergreen.

Ritz, Christian, Florent Baty, Jens C. Streibig, and Daniel Gerhard (2015). "Dose-response analysis using R". In: *PLOS ONE* 10.12. Ed. by Yinglin Xia, e0146021. ISSN: 1932-6203. DOI: 10.1371/journal.pone.0146021.