IMPACTS OF ATMOSPHERIC POLLUTANTS ON ECOSYSTEMS AND HUMAN HEALTH

Editors
Prof. Shyamal Kumar Majumdar
&
Prof. Badal Bhattacharya

INSTITUTE OF ECOTOXICOLOGY AND ENVIRONMENTAL SCIENCES

IMPACTS OF ATMOSPHERIC POLLUTANTS ON ECOSYSTEMS AND HUMAN HEALTH

Editors
Prof. Shyamal Kumar Majumdar
&
Prof. Badal Bhattacharya

Published by
INSTITUTE OF ECOTOXICOLOGY AND ENVIRONMENTAL SCIENCES
Y/7, XI, Purbachal, Salt Lake, Kolkata-700097, West Bengal, INDIA

City Office
C/O. Dr. B. Bhattacharya, 6C, 2 B, Tower No. 1. Greenwood Park Extension,
New Town, Kolkata – 700156, INDIA Phone No. +91-33-23242618, 9830348789
E-mail badalbhattacharya@hotmail.com Website: http://www.iees.org.in

Published by

INSTITUTE OF ECOTOXICOLOGY AND ENVIRONMENTAL SCIENCES
Y/7, XI, Purbachal, Salt Lake, Kolkata-700097, West Bengal, INDIA

ACKNOWLEDGEMENT

Council of Scientific and Industrial Research, New Delhi

Gas Authority of India Limited, New Delhi

Coal India Limited, Kolkata

Copywrite
Editor-in-Chief, Prof. Dr. Badal Bhattacharya, President,
Institute of Ecotoxicology and Environmental Sciences
E-mail: badalbhattacharya@hotmail.com
Website:http://www.iees.org.in
ISBN: 978-81-928924-3-6

Printed by

MUDRAKAR
18A Radhanath Mullick Lane, Kolkata - 700 012

Price
r 650.00
US $ 30

DISCLAIMER : Institute of Ecotoxicology and Environmental Sciences or any of its Editors assume any responsibility for authenticity or reliability of scientific data published in this book. The publications carried out in this book are accepted on good faith

CONTENTS

Chapter I:
Brominated Flame Retardants in Consumer Products: Benefits and Risks- from consumer products — 11
O.J.Okonkwo, K Kefeni and O Olukunle

Chapter 2:
Hexabromocyclododecane (HBCDD)- A Hazardous Flame Retardant Used in Polystyrene Building Materials — 25
Allan Astrup Jensen

Chapter 3:
Turning Beauty into the Beast: Pharmaceuticals and Personal Care Products in the Aquatic Environment — 35
Olga I. Kalantzi and Panayotis A. Siskos

Chapter 4:
Epigenetic Response to Environmental Pollutants and its Effects on Human Health: A Review — 43
Spencer Keene, Paul McFadden and S.K. Majumdar

Chapter 5:
Impact of Heavy Metal Pollution on Freshwater *Coelatura* species (*Mollusca: Bivalvia: Unionidae*) and the Ecosystem of the River Nile In Egypt — 53
Faiza M El Assal, Salwa F Sabet, Kohar G Varjabedian and Mona F Fol

Chapter 6:
A New Possibility for Separate Identification of Organophosphates and Carbamates — 81
Tonkopii V

Chapter 7:
A Lab-scale investigation into the potential polybrominated diphenyl ethers and trace metals compellation in aqueous media 89
Adegbenro P. Daso, Jonathan O.Okonkwo, Patricia B. C. Forbes and Egmont R. Rohwer

Chapter 8:
Diesel Toxicity and Soil Remediation 101
P. Agamuthu and A. Dadrasnia

Chapter 9:
Pesticide Residue Evaluation in Soil Environment of Mustard Crop using Chromatographic Techniques 113
S.P. Singh, Y.P. Singh, N.S. Bhogal and Sandeep Kumar

Chapter 10:
Study of the Genetic Mechanisms of Drug Resistance in M*ycobacterium tuberculosis* Carried Out in a Multispeciality Hospital in East Kolkata 119
Indranil Roy, Ria Saha, Debasree Mazumder and Tapan Kumar Ghosh

Chapter 11:
Remediation of Kolkata Sewage Through Waste Water Fishery and Open Circulation: A case Study 131
Jyotiskona Barik, Debabrata Mukherjee, Mrinmoy De, Shreyasee Roy and Gourab Choudhury

Chapter 12:
Loss of biodiversity due to Impact of Prawn Seed Collection-A crucial Practice of Fisheries Industry in Indian Sundarbans 141
Mayukh Barik, Shreyasee Roy, Mrinmoy De, Gourab Choudhury, Jyotiskona Barik and Debabrata Mukherjee

Chapter 13:
Assessment of plastics as refuge for aquatic organisms in urban ponds 151
Amritangshu Roy

Chapter 14:
Isolation of Porin from *Shigella dysenteriae* type1 to establish it as a Vaccine component 159
Deb Singha Roy

Chapter 15:
Present Status of Tidal Variations in the Sundarbans Estuary, India 171
Jayanta Sutradhar and Debabrata Mukherjee

Chapter 16:
Biodiesel from Algae: A Potential from Sundarban 177
Shreyasee Roy, Mayukh Barik, Debabrata Mukherjee, Gourab Choudhury and Jyotiskona Barik

Chapter 17:
Possible bio-mining by metal tolerant bacteria 185
Rajanya Bhattacharyya, Monica Ghosh, MoupiyaGiri, Ankita Sen and Debjani Dutta

Chapter 18:
Eco-tourism in Generation of Livelihood & Environmental Management in District of Purulia, West Bengal, India 195
Debabrata Mukherjee, Mrinmoy De, Jyotiskona Barik, Shreyasee Roy, Gourab Choudhury, Mayukh Barik and Aditi Bose

Chapter 19:
Stack Farming: A Step Ahead Towards Food Security 203
Gourab Choudhury, Jyotiskona Barik, Debabrata Mukherjee, Mayukh Barik, Shreyasee Roy, Aditi Bose and Mrinmoy Dey

PREFACE

The book contains a compilation of selected papers presented at the 4th International Conference on Ecotoxicology and Environmental Sciences (ICEES-2014), held in New Delhi, India in February 2014. Like the other three former meetings began in 2007, The Institute of Ecotoxicologyand Environmental Sciences based in Kolkata organized the ICEES-2014 international event. After taking a great care in the compilation process, the Institute is proud to present this edited volume to our readership containing nineteen reviewed articles from over 60 plus papers presented at the ICEES-2014 international conference. International experts authored eight of these chapters.

Environmental Ecotoxicology is a multidisciplinary field of science concerned with the study of the harmful effects of various biological and physical agents on living organisms at the population and ecosystem levels. In ecosystems, all living (animals, plants and microbes) and nonliving (air, water and soils) things are mutually interconnected and live together as a system in that environment. The sources of environmental contaminants are diverse and they are largely generated by human activities such as waste product discharge in the form of liquid, gas and fluid into the atmosphere from industrial factories, agricultural products, incinerators, sewage plants, etc. Many of these contaminants are known to devastate the ecosystems and adversely impact human health and a substantial numbers of these pollutants are known mutagenic, teratogenic or carcinogenic substances. Furthermore, the damaging effects may not only be temporary but may also have long-term effects.

In this volume, the authors address a variety of issues concerning the adverse impacts of atmospheric contaminants on ecosystems and human health. Identification, management, reduction of contaminants discharge from the sources as well as pollutants effects on environment and health are covered in several chapters, while remediation, reutilization of waste products as well as regulations and monitoring the release of waste materials into the environment are included in other chapters.

Because of the complexity and multidisciplinary nature of the subject, both national and international collaborations are relevant in order to exchange ideas among experts on ecotoxicology and related subjects. International conference like the one organized by The Institute of Ecotoxicology and Environmental Sciences held in New Delhi in 2014 and other previous ones not only enhance interest in ecotoxicology research but also foster national and international scientific collaborations. The organizers of the next ICEES-2016 conference in Kochi, India in February 2016 have been planning to have a higher level of engagement involving more international experts in the areas of ecotoxicology and environmental sciences.

The book will be of interest to a wide audience including ecotoxicologists, environmental scientists, professionals, educationists, undergraduate and graduate students as well as interested public since adverse effects of environmental pollutants on ecosystems and human health are universal problems. The editors express their appreciation to the contributors for their quality work and cooperation. Gratitude is extended to The Institute of Ecotoxicology and Environmental Sciences for organizing the Delhi event and to Ms. Shreyasee Roy of St. Xavier's College, Kolkata and others who were directly or indirectly involved in the preparation of this volume.

Any findings, opinions, conclusions, or recommendations expressed in the book are those of the authors and do not reflect the views of the editors or The Institute of Ecotoxicology and Environmental Sciences. Likewise, the editors and the Institute cannot take any responsibility or liability for errors and misrepresentation of data that may contain in the book.

Prof. Shyamal K. Majumdar, Ph. D. Prof. Badal Bhattacharya, Ph.D.
August, 2015

Chapter 1

Brominated flame retardants in indoor office dust samples from consumer products

***O.J Okonkwo, K Kefeni, O Olukunle**
Department of Environmental, Water & Earth Sciences, Faculty of Science,
Tshwane University of Technology, 175 Nelson Mandela Drive,
Arcadia Pretoria, South Africa
***Corresponding author:**OkonkwoOj@tut.ac.za

Abstract

Of the 32 congeners considered for identification, (BDE 3, 15, 17, 28, 47, 66, 77, 85, 99, 100, 126, 138, 153, 154, 183, 209 and BB-1, 2, 4, 10, 15, 26, 29, 30, 31, 38, 49, 80, 103, 153, 155 and 209) only 6 from each group were detected. The concentration å$_6$PBDEs detected in office dust (BDE17, 66, 85, 99, 153 &209) ranged between 33-1510 ng g^{-1} dry weight (dw) with a median and mean of 452 and 478 ng g^{-1}dw, respectively. The å$_6$PBBs concentration detected (BB2, 4, 15, 30, 153, and 209) ranged between <dl-271 ng g^{-1}dw with a median and mean of 61 and 92 ng g^{-1}, respectively. Both BB4 and BDE99 were frequently detected in 76% of the samples studied. A Spearman rank correlation between å$_6$PBBs and å$_6$PBDEs (r_s= 0.69, p = 0.0008), indicated a statistical significant positive correlation for the similarity of pollution sources for both compound classes. However, poor correlation was observed between the number of electronic materials and summation of concentrations of PBDEs and PBBs congeners detected. Concentrations of PBDEs detected in this study are substantially lower than reported in office dust in other countries.

The concentrations of PBDEs congeners detected in office dust are substantially lower than reported in office dust in developed countries. The low distribution of BFRs concentration detected in the current study which ranged from none to low highlights the fact that South Africans, particularly those living in Pretoria are exposed to low concentrations of PBDEs and PBBs.

1. Introduction

Polybromobiphenyls (PBBs) and polybromodiphenyl ethers (PBDEs) are among BFRs commonly used as additive flame retardants (Alaee *et al.*, 2003; de Wit *et al.*, 2010). Despite their social benefits, they are well-known environmental pollutants and can easily be released from the materials they have been added and tend to settle on dust particles (Schecter *et al.,* 2009). Therefore, concerns for these emerging chemicals have risen because they have become

ubiquitous and toxic to humans and animals (Birnbaum & Staskai, 2004; Hites, 2004). Their adverse health effects such as on endocrine systems, thyroid hormones, neurobehavioral development, and also causes of cancer have been reported (Branchi *et al.*, 2003; Darnerud, 2003; Herbstman *et al.*, 2010; Kefeni *et al.*, 2011b). Looking into the consequence some of the countries voluntarily phased out the production and usage of these chemicals, however; Deca-mixture is still used in most of the countries.

To date, published data on the presence of PBBs and PBDEs in indoor dust from consumer goods such as electronic equipment, carpets and padded chairs in developing countries is scarce. Therefore, the main objectives of this research were: (1), to identify and quantify PBBs and PBDEs congeners that may be present in office dust in some parts of South Africa. (2), to examine the correlation between PBBs and PBDEs concentration in settled office dust and office dust attributes.

2. Experimental

2.1 Chemicals and reagents

Dust standard reference material - 2585 purchased from National Institute of Standards and Technology (Gaithersburg, MD, USA). 1.2 ml of 50 mg L^{-1} of each certified standard solutions of sixteen PBDEs congeners (BDE-3, 15, 17, 28, 47, 66, 77, 85, 99, 100, 126, 138, 153, 154, 183, and 209) and sixteen PBBs congeners (BB-1, 2, 4, 10, 15, 26, 29, 30, 31, 38, 49, 80, 103, 153, 155, and 209) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). 1.2 mL of 50 mg L^{-1} isotopic labelled internal standard$^{13}C_{12}$-BDE-139 and $^{13}C_{12}$-BDE-209 were purchased from Cambridge isotope laboratories (CIL, Andover, MA, USA). Commercial decabromodiphenyl ether mixture that contains > 95% Decabromodiphenyl ether (BDE-209) manufactured by (FlukaChemie GmbH, CH - 9471 Buchs, EC No: 2146049, Switzerland) was purchased. Copper powder (purity 99.98% from Saarchem (Pty) Ltd., Muldersdrift, South Africa), silicagel (100 - 200 mesh), sodium sulphate (purity 99.9%), glass wool and HPLC grade solvents: acetone, hexane, dichloromethane, ethanol and toluene; products of Sigma Aldrich (Chemie GmbH, Steinheim, Germany), 50 mL of nonane (Purity 99.8%, Sigma Aldrich, product of Switzerland) were purchased from Industrial Analytical Pty. Ltd. Midrand, Gauteng, South Africa.

2.2 Sampling and pre-sample preparation

Dust samples were collected from offices using surface wiping and suction with a vacuum cleaner of some staff members at Tshwane University of Technology, Pretoria. The selection of offices was based on the willingness of the staff members.

2.3 Sample treatment and instrumentations

The extraction of all dust samples wascarried out using Soxhlet extraction throughout this work. In each case, dry dust sample was weighed, 0.25 g of pre-prepared activated copper powder was added (for removal of elemental sulphur) and the mixture homogenized, transferred to a cellulose extraction thimble (19 mm ID and 90 mm in length), covered with glass wool, placed inside Soxhlet apparatus and extracted with 250 – 270 mL of *n*-hexane: acetone (2:1, v/v) for 8 h. Thimbles containing activated copper powder and glass wool that represented the method blank samples were also extracted under the same condition along with the

samples.All dust extract were cooled and reduced to about 2 mL in a rotary evaporator (RotaVapor R- 210, BÜCHI Labortecnik AG, Switzerland) under a fume cupboard. The temperature of the water bath of the rotary evaporator was adjusted to 40°C in order to reduce the loss of sample. For clean up, a Pasteur pipette column of 5 mm inner diameter was plugged with glass wool at the bottom and packed with pre-prepared silica gel and sodium sulphate from bottom to top in the following order: neutral silica gel (0.2 g), basic silica gel (0.2 g), neutral silica gel (0.2 g), acidic silica gel (0.2 g) and sodium sulphate (0.2 g) at the top. To enhance cleaning, glass wool was used for partitioning in between each packed chemicals. Each of the packed disposable Pasteur pipette columns was first eluted with 20 mL of *n*-hexane: dichloromethane (5:1, v/v) mixture after which the extract was transferred onto it. Subsequently, it was eluted with 2x10 mL of *n*-hexane: dichloromethane (5:1, v/v) mixture. The extract was further concentrated under a gentle flow of nitrogen to about 50 μL. Finally, the concentrated extract was diluted to 200-250 μL by a mixture containing *n*- nonane: toluene (9:1, v/v*)* and ready for analysis.

3. Instrumentation

The dust extracts were analysed using an Agilent 7890A GC system (serial number: US 92023178, made in USA). One μL solutions of the extracted sample were injected by Agilent Technologies 7693 Autosampler into split/splitless injection port on DB-5 GC column (30 m, 0.25 mm ID, 0.10 μm d_f). The GC was coupled to an Agilent 5975C inert MSD with triple axis detector, operated in EI mode. Operating conditions were as follows: ion source of 250°C, and transfer line of 300 °C. Identifications was carried out using full scan mode by monitoring the presence of the mass spectra of molecular ion and two qualifier ions of each congener at the elution retention time. Each congener was quantified against five level external standard calibration curves. BB-209 and BDE-209 were analysed separately using ZB-5 GC column (15 m, 0.25 mm I.D., 0.25 μm d_f) with similar oven programme except the final hold time which was changed to 3 min. Nitrogen was used as a carrier and makeup gas with a flow rate of 2.5 and 30 mL min^{-1}, respectively.

4. Determination of LOD and LOQ

Throughout this work, a signal-to-noise ratio (S/N) of 3:1 and 10:1, were used to determine the LOD and LOQ, respectively. However, BDE-209 was detected in the blank samples during the application of the optimized method on real sample analysis (section 4.2.6.1.2). Hence, the blank determination methods were used for the estimation of LOD and LOQ values.

5. Quality control/quality assurance

Several quality control methods were assessed in order to obtain reliable data for each analysis. Glass wool, silica gel and sodium sulphate were baked in the oven to remove any volatile organic compounds and some other impurities. Silica gel and sodium sulphate were stored in glass jar which was pre-cleaned and rinsed with n-hexane:acetone solvent and then sealed. Glass wool was wrapped with aluminium foil and kept inside a desiccator to avoid absorption of moisture. All glassware used in this study were cleaned with ultrapure water and finally rinsed with n- hexane:acetone mixture and dried. Furthermore, the method performance and validation was evaluated by extracting organic contaminants in house dust “SRM – 2585”.

6. Results and discussion

Chromatograms of thirty-two standards with concentration of 0.5 ng μL^{-1} and flow rate of 1.5 mL min^{-1}of nitrogen gas on GC-ECD using ZB-5 (30m, 0.25 mm ID, 0.1 μm d_f) column is given in Figure 1.

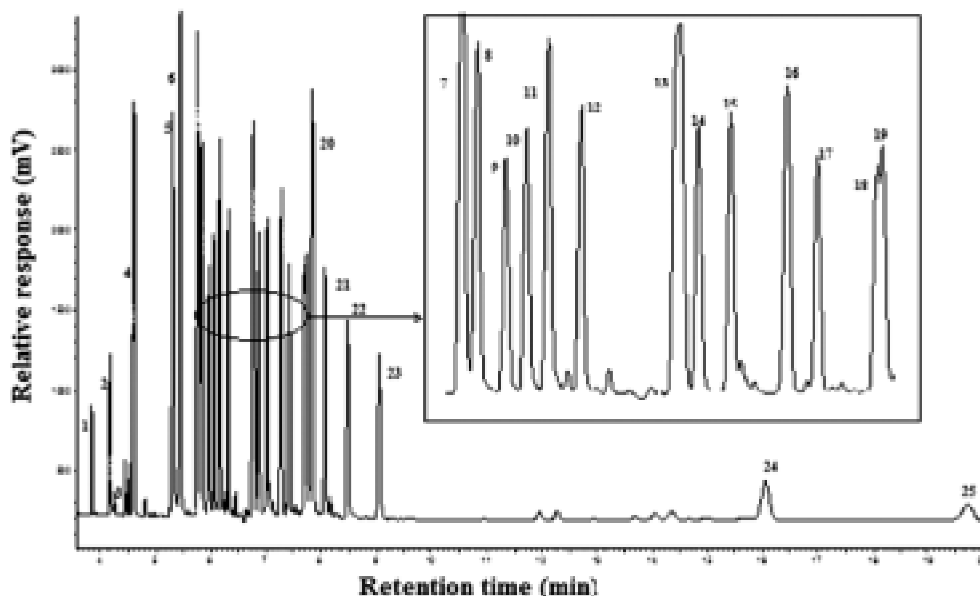


Figure1:GC-ECD Chromatograms of 32 standards on ZB-5 column (30 m, 0.25 mm ID, 0.1 μm d_f): 1) BB- 1, 2) BB-2, 3) BDE-3, 4) BB-4 & 10, 5) BB-15 & BDE-15, 6) BB-30, 7) BB-29 &26, 8) BB-31, 9) BDE-17, 10) BDE- 28, 11) BDE-38, 12) BB-49, 13) BDE-47, BB -80 & BB-103, 14) BDE-66, 15) BDE-77, 16) BDE-100 & BB-155, 17) BDE-99, 18) BDE-85, 19) BDE-126, 20) BDE-154 & BB-153, 21) BDE-153, 22) BDE-138, 23) BDE-183, 24) BB-209, 25) BDE-209.

6.1 Recovery test using different solvents

The most preferred solvents for extraction were selected based on the recovery obtained from the extraction of 1.0 g dust standard reference material “SRM–2585”. The result showed no significant difference among the mixture of different combination of HPLC grade solvents as given in Table 1. However, to some extent good recovery of BDE-209 was obtained when hexane:acetone (2:1, v/v) was used while very low recovery of the same congener was obtained using hexane:toluene. Alsohexane:acetone (2:1, v/v) showed a recovery of about 104%. Consequently, throughout this work, the mixture of hexane:acetone (2:1, v/v) was used. On the whole, irrespective of the type of solvents used, about 96% of the congeners were recovered. This showed that a good agreement between certified and measured values. Higher recovery for the three congeners (BDE-17, 28 and 66) were observed. This observation was partially attributed to small contribution from debromination during extraction, analysis or more likely the breakthrough in the silica gel column during clean up because of the non-retention in the column unlike the more brominated congeners.

Table 1: Mean percentage recovery ± SD of measured organic contaminants in house dust "SRM- 2585" relative to the certified values using different solvents

PBDEs congener	Hexane:acetone (2:1, v/v)	Hexane:acetone (2:1, v/v)	Hexane:toluene (3:1, v/v)e	Hexane:dichloromethane (2:1, v/v)
BDE-17	137 ± 7.9	106 ± 5.3	134.3 ± 25	108 ± 3.8
BDE-28	132 ± 15	108 ± 11	132 ± 11	103 ± 1.6
BDE-47	89 ± 11	94 ± 11.6	80 ± 10	84.3 ± 10
BDE-66	131 ± 22	103 ± 1	102.9 ± 10.2	111 ± 13
BDE-85	104 ± 14	94.4 ± 4.8	83.6 ± 1.5	105 ± 14
BDE-99	85 ± 2.6	90 ± 3.6	85.3 ± 5.9	89 ± 7
BDE-100	90 ± 5.0	82 ± 7.8	90.6 ± 1	92.3 ± 1
BDE-138	97 ± 38	101 ± 37	72 ± 15	88 ± 28
BDE-153	102 ± 4.7	96.4 ± 7.2	102.4 ± 12	103 ± 3.3
BDE-154	98 ± 3.4	91 ± 16	98 ± 26	102 ± 21
BDE-183	94 ± 1	87 ± 20	66 ± 2.3	86 ± 30
BDE-209	84 ± 5.7	80.8 ± 5.7	55 ± 1.8	75 ± 3.4

6.1.1 Quality control/ Quality assurance

The concentration of calibration curves for each standard ranged between 0.02-1.00 ng μL^{-1}, showed good linearity in the ranges considered with regression coefficient (r^2 e" 0.99). Furthermore, none of the congeners were detected in solvent blank and method blank. Therefore, the LOD was calculated by extrapolating the concentration that would give a signal-to-noise ratio of 3 (S/N=3) by injecting extracted spiked sample of lowest concentration. Except for BB-209 and BDE-209 with LOD of 0.8 ng g^{-1} and 1.2 ng g^{-1} respectively, the LOD values for other PBBs and PBDEs congeners ranged from 0.3–0.5 ng g^{-1}. Good recoveries of the SRM-2585 indicated the high quality of the method. The precision of the method can be seen from low standard deviation obtained for most of the congeners which is less than 10% showing better repeatability.

6.1.2 Levels and profiles of PBBs and PBDEs in office dust

From a total of thirty two target congeners measured, sixteen from each PBDEs (BDE-3, 15, 17, 28, 47, 66, 77, 85, 99, 100, 126, 138, 153, 154, 183, and 209) and PBBs congeners (BB-1, 2, 4, 10, 15, 26, 29, 30, 31, 38, 49, 80, 103, 153, 155, and 209), only five and six were detected from PBBs (BB-2, 4, 30, 153 and 209) and PBDEs (BDE-47, 66, 85, 99, 153 and 209), respectively. The summary of the analysis results for all eleven detected individual congeners, excluding all the twenty-one non-detected congeners, are shown in Table 2. From the analysis result, only two congeners (BDE-47 and BDE-99) had a median concentration above detection limit and detected in greater than 50% of the samples. The detection frequency was dominated by BDE-99 (81.3%) and followed by BDE-47 (62.5%). The åPBDEs of the six congeners detected ranged between 21.4 and 578.6 ng g^{-1} with a mean and median

concentration corresponding to 169 and 162 ng g^{-1} respectively. A closer examination of each dust concentration indicated irregular distributions of PBDEs. This can be seen from high value of SD (144.5 ng g^{-1}) of the summation of the six congeners. This may have been due to the difference in electronic material, floor type, frequency of cleaning and ventilation conditions of each offices. The highest detected concentration for PBDEs corresponds to 578.6 ng $g^{-1}$of BDE-209. This dust sample was collected from an office that contains many old computers, sofas, padded chairs and some other electronic materials. For PBBs, the most frequently detected congener was BB-4 (43.8%) followed by BB-2 (31.3%) of the studied samples. The Ó$_5$PBBs detected congeners ranged from <dl - 196 ng g^{-1} with mean and median concentration of 38.2 and 11.4 ng g^{-1}. As expected, the concentrations of PBBs detected were relatively very low due to the less use and ban of its production compared to that of PBDEs.

According to *Hale et al. (2001),*penta-mixture mainly composed of five congeners, BDE-99, 47, 100, 153 and 154 in the ratio of 12:9:2:1:1. Thus, BDE-99 and BDE-47 almost share about 48% and 36% of the penta-mixture, respectively. On the other hand, different percentage composition of BDE-47 and BDE-99 in the penta-mixture (Bromkal 70-5DE); have been reported. *Sjodin et al. (1998) reported*37% (BDE-47) and 35% (BDE-99). These results show the composition of these products may vary with manufacturer or due to the difference between batches of production (Gevao *et al.,* 2006). In fact, in this study lower concentration of BDE-47 was detected compared to BDE-99. The lower congeners such as BDE-3, 15, 17, and 28 which were not detected may be due to frequent cleaning, adequate ventilation and the frequent use of fan in the offices. The absence of some higher PBDEs may be attributed to their low concentration in the environmental samples below the detection limits of the instrument.

In Table 2, samples 13, 14, 15 and 16 were dust samples collected using a vacuum cleaner and the rest by wiping surfaces with glass wool. The average concentrations of PBBs analysed in dust samples collected by vacuum cleaner (82.7 ng g^{-1}) were found to be higher than that collected using glass wool (23.4 ng g^{-1}). This was attributed to the high concentration of BB-209 detected in sample number fourteen that changed the statistical data. For PBDEs, slightly higher average concentration å$_6$PBDEs was observed in dust collected using glass wool (171 ng g^{-1}) than vacuum cleaner (163 ng g^{-1}). The low concentration of BFRs in dust collected by vacuum cleaner can be attributed to the dilution of the congeners by higher dust loadings. This means that the higher the dust loading of no BFRs present, the more likely it is to detect lower concentrations of PBDEs congeners. Besides lower dust loading, dust collection through wiping using glass wool targets only the surface of materials like computers, printers, fans, air conditioner units, chairs and tables where the sources of BFRs are expected and easier for collection; therefore, this may contribute to increase in the concentration of BFRs.

Table 2: Summary of PBBs and PBDEs concentration (ng g^{-1}) detected in office dust

Sample no	BB-2	BB-4	BB-30	BB-153	BB-209	≅PBBs	BDE-47	BDE-66	BDE-99	BDE-85	BDE-153	BDE-209	©PBDEs
1	<dl	32.0	<dl	<dl	<dl	32.0	53.0	35.0	88.9	40.3	<dl	<dl	217.2
2	31.7	45.7	<dl	<dl	<dl	77.4	<dl	<dl	<dl	7.6	<dl	571	578.6
3	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	21.4	<dl	<dl	21.4
4[a]	22.2	<dl	<dl	<dl	<dl	22.2	44.0	<dl	50.0	<dl	<dl	2.8	96.8
5	<dl	<dl	<dl	<dl	126	126	44.0	44.9	89.9	<dl	<dl	142	320.8
6	<dl	<dl	<dl	<dl	<dl	<dl	78.9	<dl	127.7	44.7	<dl	<dl	251.3
7	<dl	<dl	15.3	<dl	<dl	15.3	<dl	<dl	<dl	44.4	12.5	<dl	56.9
8[a]	<dl	<dl	<dl	<dl	<dl	<dl	14.8	<dl	75.0	<dl	<dl	<dl	89.8
9[a]	<dl	<dl	<dl	<dl	<dl	<dl	62.7	<dl	82.8	<dl	<dl	<dl	145.5
10[b]	<dl	<dl	<dl	<dl	<dl	<dl	46.9	<dl	78.1	<dl	<dl	87.5	212.5
11[b]	<dl	7.5	<dl	<dl	<dl	7.5	<dl	<dl	32.5	<dl	<dl	<dl	32.5
12[b]	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	31.6	<dl	<dl	<dl	31.6
13[c]	7.9	34.2	<dl	5.4	<dl	47.5	61.8	<dl	119.7	<dl	<dl	<dl	181.5
14[c]	26.5	26.5	<dl	<dl	143	196	76.7	<dl	109.8	<dl	<dl	<dl	186.5
15[c]	33.3	33.3	<dl	19.4	<dl	86.0	81.9	<dl	120.8	12.5	<dl	39.0	254.2
16[c]	<dl	1.3	<dl	<dl	<dl	1.3	<dl	<dl	28.8	<dl	<dl	<dl	28.8
Ó16PBB/ PBDE	121.6	180.5	15.3	24.8	269.0	611.2	564.7	79.9	1035.6	170.9	12.5	842.3	2706
mean	7.6	11.3	1.0	1.6	16.8	38.2	35.3	5.0	64.7	10.7	0.8	52.6	169.1
SD	12.8	16.6	3.8	4.9	46.0	56.8	32.4	13.8	44.9	17.2	3.1	144.0	144.5
Median	<dl	<dl	<dl	<dl	<dl	11.4	44	<dl	76.5	<dl	<dl	<dl	162.2
Min	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	21.4
Max	33.3	45.7	15.3	19.4	143	196	81.9	44.9	127.7	44.7	12.5	571	578.6
%ÓPBB/PBDE	19.9	29.5	2.5	4.1	44.0		20.9	3.0	38.3	6.3	0.5	31.1	
n> dl	5	7	1	2	2		10	2	13	6	1	5	
%det.fre	31.3	43.8	6.3	12.5	12.5		62.5	12.5	81.3	37.5	6.3	31.3	

<*dl* = less than detection limit, det. fre = detection frequency, [a] pooled samples collected from three offices, [b] pooled samples collected from two offices, [c] Samples collected using vacuum cleaner

Concerning sources of BFRs, for both PBBs and PBDEs Spearman rank correlation coefficients were computed between the number of electronic materials used in the office and concentration of å$_5$PBBs and å$_6$PBDEs detected. Nearly, no correlation was observed for both å$_5$PBBs (r_s = -0.26, *p* = 0.07) and Ó$_6$PBDEs (r_s = 0.07, *p* = 0.0004). Similarly, studies on PBDE concentrations in dust and residential characteristics (i.e., the number of televisions, computers, and other electronics in the household) found no significant correlations (Chen *et al.*, 2008; Tan *et al.*, 2007; Wu *et al.*, 2007). Comparison of the analysis results against information recorded during dust sample collection showed no uniform results. It was, therefore, a difficult task to identify exactly the main sources. However, it is highly probable that the BFRs may have originated from the materials (computers, printers, carpets, sofas and other electronic) found in the offices. From the Spearman rank correlation, calculation between concentration of å$_5$PBBs and å$_6$PBDEs (r_s = 0.55, *p* = 0.003), a significant positive correlation was observed; this shows a common pollution source for both BFRs. Similarly, a significant strong correlation between the two frequently detected congeners BDE-47 and BDE-99 (r_r = 0.92, *p* = 0.04) was found which also supports the common emission sources. As shown in Table 3, few congeners have significant spearman's correlation coefficients.

Table 3: Spearman's rank correlation coefficients and the corresponding *p*-value for the analysed PBBs and PBDEs

Congeners	BB-4	BB-30	BB-153	BB-209	BDE-47	BDE-66	BDE-99	BDE-85	BDE-153	BDE-209
BB-2	0.6 *8 0.49	-0.16 0.06	0.53 0.09	0.20 0.45	0.31 0.00	-0.23 0.58	0.13 0.00	-0.19 0.57	-0.16 0.05	0.47 0.23
BB-4		-0.18 0.49	0.45 0.06	0.07 0.09	0.27 0.45	0.06 0.00	0.22 0.58	0.03 0.00	-0.18 0.57	0.50 0.05
BB-30			-0.08 0.71	-0.10 0.19	-0.29 0.00	-0.10 0.27	-0.38 0.00	0.52 0.04	1.00 0.89	-0.10 0.17
BB-153				-0.12 0.21	0.44 0.00	-0.12 0.36	0.42 0.00	-0.02 0.06	-0.08 0.60	-0.05 0.18
BB-209					0.31 0.20	0.45 0.34	0.31 0.01	-0.24 0.62	-0.10 0.18	0.04 0.36
BDE-47						0.15 0.00	0.92 0.04	0.08 0.01	-0.2 9 0.00	-0.23 0.64
BDE-66							0.21 0.00	0.16 0.31	-0.10 0.25	0.07 0.21
BDE-99								-0.02 0.00	-0.38 0.00	-0.31 0.75

BDE-85	0.52	-0.11
	0.04	0.26
BDE-153		-0.10
		0.17

* Each cell contains Spearman correlation coefficient and *p*-value

6.1.3 Human exposure Rates

Rough estimation of the daily intake of PBDEs in young children and adults via house dust on the basis of the average concentration of total PBDEs measured in house dust can be made based on low and high dust ingestion scenarios. However, in addition to its high uncertainty nature of estimation of exposure rates, there are some limitations to make comparison of exposure rates with different published data, probably due to three main reasons. First, there is no available standard average dust ingestion figure in the literature. For example, for adults the USEPA,recommends 0.56 mg day^{-1} and 110 mg day^{-1} of low and high dust ingestion rates, respectively (USEPA, 1997). Similarly, the average and high daily dust ingestion rate recommended for adults and toddlers is 20 and 50 mg day^{-1} and 50 and 200 mg day^{-1}, respectively (Jones-Otazo *et al.,* 2005).On the other hand, the assumption of mean daily dust ingestion of 4.16 and 50 mg day^{-1} and high dust ingestion rate of 100 and 200 mg day^{-1} for adults and children (6 months to two years), respectively have been used (Wilford *et al.,* 2005). Secondly, the metrics of presentation of results for toxicant or congeners detected are surface loading (mass of the congeners per square meters) or surface concentration (mass of the congener per mass of dust used for analysis) which is equally important (Lioy *et al.,* 2002). Thirdly, irrespective of the indoor type (office, house or hotel) an average indoor exposure rates are used by some researchers, for instance Kang *et al.* (2011) presented the exposure rate of work place and home together. This means that researchers use different average exposure values, units or total exposure rates. Therefore, such non- uniformity of data presentation may limit comparison with most published data. A summary of the daily average dust ingestion obtained in this study and comparison with similar results from other study reports are shown in Table 4. The calculation was done assuming 100% absorption of intake, and mean adult and toddlers ingestion of 20 and 50 mg day^{-1} and high dust ingestion rate of 50 and 100 mg day^{-1}(Harrad *et al.*, 2008b; Jones-Otazo *et al.*, 2005). Using the median and mean concentration of BDE-209 and åPBDEs in house dust, the mean and high dust ingestion rate for adults and toddles were calculated. Accordingly, for toddlers the median value exposure rate of BDE-209 and åPBDEs ranged from 0.05–0.18 and 0.61–2.44 ng day^{-1}, while for adults it ranged from 0.02–0.05 and 0.24–0.61 ng day^{-1}, respectively. Similarly, the mean values ranged from 1.75–6.98 and 0.81–3.24 ng day^{-1}; and 0.7–1.75 and 0.32–0.81ng day^{-1} for adults and toddlers, respectively. Furthermore, from the data in Table 4, the comparison of adult exposure rate to PBDEs in both microenvironments using mean and high dust ingestion rate showed human exposure to PBDEs in office, was about 9 and 2 times greater than house, respectively (irrespective of median or mean used for calculation). Similarly, compared with other studies, the mean and high daily dust ingestion exposure rates estimated

from this study was 1 to 2 and 2 to 3 orders of magnitude lower than the developed countries, respectively. Therefore, this study provides the first report on the exposure rates of PBDEs to South Africans living in Pretoria.

The low distribution of BFRs concentration detected in different houses, which ranged from none to low highlights the fact that South Africans, particularly those living in Pretoria are exposed to low concentrations of PBDEs and PBBs from house dust compared to results from other countries. Such study should be extended to all regions of South Africa for determination of the overall exposure rates for the whole population. To our knowledge, there is no data and exposure rate determination in most Africa countries to BFRs. Therefore, studies should be conducted to investigate such contaminants in other African countries where data regarding environmental levels of BFRs and human exposure rates are completely lacking.

Table 4: Summary of estimate of exposure (ng day^{-1}) of adult and toddlers to PBDEs via home dust ingestion in this study and other selected studies.

Country	Exposure group	BFRs	Mean dust ingestion rate		High dust ingestion rate		References
			median	mean	median	mean	
South Africa[c]	Toddlers	åPBDEs[a]	0.61	1.75	2.44	6.98	this study
		BDE-209	0.05	0.81	0.18	3.24	
	Adult	åPBDEs[a]	0.24	0.70	0.61	1.75	
		BDE-209	0.02	0.32	0.05	0.81	
South Africa[d]	Adult	åPBDEs[b]	2.19	2.33	5.48	5.83	(Kefeni and Okonkwo, 2012)
		BDE-209	<dl	1.052	<dl	2.63	
Canada	Toddlers	åtri- hexa-BDEs	31	55	120	220	(Harrad *et al.,* 2008b)
		BDE-209	28	33	110	130	
	Adult	åtri- hexa-BDEs	12	22	31	55	
		BDE-209	11	13	28	33	
New Zealand	Toddlers	åtri- hexa-BDEs	4.8	8.1	19	32	(Harrad *et al.,* 2008b)
	Adult	åtri- hexa-BDEs	1.9	3.2	4.8	8.1	
UK	Toddlers	åtri- hexa-BDEs	2.9	4.9	12	20	(Harrad *et al.,* 2008b)
		BDE-209	140	2200	560	9000	
	Adult	åtri- hexa-BDEs	1.2	2	2.9	4.9	

		BDE-209	56	900	140	2200	
US	Toddlers	åtri- hexa-BDEs	82	150	330	590	(Harrad *et al.,* 2008b)
		BDE-209	65	80	260	320	
	Adult	åtri- hexa-BDEs	33	59	82	150	
		BDE-209	26	32	65	80	
		BDE-209	26	32	65	80	

[a]å(BDE-3, 15, 47, 66, 100, 99, 85, 154, & 153), [b] å(BDE-47, 66, 99, 66, 85, & 153), [c] = house, [d] = office

7. Conclusions

This study was the first of its kind conducted in South Africa that provides the concentration and compositional profiles of PBBs and PBDEs in office dust. The concentrations of PBDEs congeners detected in office dust are substantially lower than reported in office dust in developed countries. The low distribution of BFRs concentration detected in the current study which ranged from none to low highlights the fact that South Africans, particularly those living in Pretoria are exposed to low concentrations of PBDEs and PBBs.

References

1. ALAEE, M., ARIAS, P., SJÖDIN, A. & BERGMAN, Å. An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. *Environment International,*2003.29(6):683-689.
2. BIRNBAUM, L. S. & STASKAI, D. F. Brominated flame retardants: Cause for concern? *Environmental Health perspective,* 2004. 112(1):9 - 17.
3. BRANCHI, I., CAPONE, F., ALLEVA, E. & COSTA, L. G. Polybrominated diphenyl ethers: neurobehavioral effects following developmental exposure. *Neurotoxicology,* 2003.24(3):449-462.
4. CHEN, L., MAI, B., XU, Z., PENG, X., HAN, J., RAN, Y., et al. In- and outdoor sources of polybrominated diphenyl ethers and their human inhalation exposure in Guangzhou, China. *Atmospheric Environment,* 2008.42(1):78-86.
5. DARNERUD, P. O. Toxic effects of brominated flame retardants in man and in wildlife. *Environment International,* 2003. 29(6):841-853.
6. DE WIT, C. A., HERZKE, D. & VORKAMP, K. Brominated flame retardants in the Arctic environment — trends and new candidates. *Science of The Total Environment,* 2010.408(15):2885-2918.
7. GEVAO, B., AL-BAHLOUL, M., AL-GHADBAN, A. N., AL-OMAIR, A., ALI, L., ZAFAR, J., et al. House dust as a source of human exposure to polybrominated diphenyl ethers in Kuwait. *Chemosphere,*2006.64(4):603-608.
8. HALE, R. C., LAGUARDIA, M. J., HARVEY, E. P., GAYLOR, M. O., MAINOR, T. M. & DUFF, W. H. Flame retardants: persistent pollutants in land-applied sludges. Nature2001. 412:141-142.
9. HARRAD, S., IBARRA, C., DIAMOND, M., MELYMUK, L., ROBSON, M., DOUWES, J., et al. Polybrominated diphenyl ethers in domestic indoor dust from Canada, New Zealand, United Kingdom and United States. *Environment International,* 2008b. 34(2):232-238.

10. HERBSTMAN, J. B., SJÖDIN, A., KURZON, M., LEDERMAN, S. A., JONES, R. S., RAUH, V., et al. Prenatal exposure to PBDEs and neurodevelopment. *Environmental Health perspective,*2010. 118(5):712-719.
11. HITES, R. A. Polybrominated diphenyl ethers in the environment and in people: A meta-analysis of concentrations. *Environmental Science & Technology,* 2004.38(4):945-956.
12. JONES-OTAZO, H. A., CLARKE, J. P., DIAMOND, M. L., ARCHBOLD, J. A., FERGUSON, G., HARNER, T., et al. Is House Dust the Missing Exposure Pathway for PBDEs? An Analysis of the Urban Fate and Human Exposure to PBDEs. *Environmental Science & Technology,* 2005.39(14):5121-5130.
13. KANG, Y., WANG, H. S., CHEUNG, K. C. & WONG, M. H. Polybrominated diphenyl ethers (PBDEs) in indoor dust and human hair. *Atmospheric Environment,* 2011. 45(14):2386-2393.
14. KEFENI, K., OKONKWO, J., BOTHA, B. & OLUKUNLE, O. Polybromobiphenyls and Polybromodiphenyl ethers in indoor dust from Pretoria, South Africa. *Organohalogen Compd,* 2011a. 73:761-763.
15. KEFENI, K. K. & OKONKWO, J. O. Analysis of major congeners of polybromobiphenyls and polybromodiphenyl ethers in office dust using high resolution gas chromatography–mass spectrometry. *Chemosphere,* 2012. 87(9):1070-1075.
16. KEFENI, K. K., OKONKWO, J. O., OLUKUNLE, O. I. & BOTHA, B. M. Brominated flame retardants: sources, distribution, exposure pathways, and toxicity. *Environmental Reviews,* 2011b. 19(NA):238-254.
17. LIOY, P. J., FREEMAN, N. C. G. & MILLETTE, J. R. Dust: A Metric for Use in Residential and Building Exposure Assessment and Source Characterization. *Environmental Health perspective,* 2002.110(10):969-983.
18. SCHECTER, A., SHAH, N., COLACINO, J. A., BRUMMITT, S. I., RAMAKRISHNAN, V., ROBERT HARRIS, T., et al. PBDEs in US and German clothes dryer lint: A potential source of indoor contamination and exposure. *Chemosphere,* 2009. 75(5):623-628.
19. SJÖDIN, A., JAKOBSSON, E., KIERKEGAARD, A., MARSH, G. & SELLSTRÖM, U. Gas chromatographic identification and quantification of polybrominated diphenyl ethers in a commercial product, Bromkal 70-5DE. *Journal of Chromatography A,*1998. 822(1):83-89.
20. TAN, J., CHENG, S. M., LOGANATH, A., CHONG, Y. S. & OBBARD, J. P. Polybrominated diphenyl ethers in house dust in Singapore. *Chemosphere,*2007. 66(6):985-992.
21. USEPA.Exposure Factors Handbook, EPA/600/P-95/002Fa; National Center for Environmental Assessment: Washington, DC,1997. Volume 1, Chapter 4.
22. WILFORD, B. H., SHOEIB, M., HARNER, T., ZHU, J. & JONES, K. C. Polybrominated diphenyl ethers in indoor dust in Ottawa, Canada: implications for sources and exposure. *Environmental Science & Technology,*2005.39:7027-7035.
23. WU, N., HERRMANN, T., PAEPKE, O., TICKNER, J., HALE, R., HARVEY, E., et al. Human Exposure to PBDEs: Associations of PBDE Body Burdens with Food Consumption and House Dust Concentrations. *Environmental Science & Technology,* 2007. 41(5):1584-1589.

Chapter 2

Hexabromocyclododecane (HBCDD)
- A Hazardous Flame Retardant Used in Polystyrene Building Materials

***Allan Astrup Jensen**
Research Director
Nordic Institute of Product Sustainability, Environmental Chemistry and Toxicology, Denmark
***Corresponding author:**allan.astrup.jensen@gmail.com

Abstract

Hexabromocyclododecane (HBCDD or HBCD) is used in large quantities as a flame retardant in building materials made of expanded- and extruded polystyrene (EPS/XPS).

In addition, HBCDD is increasingly used as a flame retardant in electronics as a substitute for the banned flame retardants: Polybrominated diphenyl ethers (PBDEs).

HBCDD is a lipophilic and persistent organic pollutant which has been detected in air, water, soil and sediments worldwide, and HBCDD is found in indoor air and dusts and contaminates building waste. HBCDD is an endocrine disruptor which accumulates in natural organisms and magnifies through the food chain, leading to progressively increasing background levels in wildlife and in human tissues, including in human milk. Human exposures are mainly from food and indoor dusts.

In May 2013 HBCDD became listed in Annex A of the Stockholm Convention. Parties of the Convention can, however, despite the availability of alternatives permit its use in EPS/XPS in buildings for further 5 years, if the content of HBCDD is marked.

Keywords

Hexabromocyclododecane, HBCDD, HBCD, brominated flame retardant, persistent organic pollutant, polystyrene insulation.

1. Introduction

Hexabromocyclododecane (HBCDD or HBCD) is an emerging chemical used mainly as a flame retardant in polystyrene-based building insulation products. It is a lipophilic and persistent organic pollutant able to bioaccumulate in organisms and biomagnify through the food chain, leading to progressively increasing background levels in human tissues and in wildlife. The extent of this accumulation correlates directly with its ever-more prevalent use. A general review has been published by the Nordic Council of Ministers (2008).

In 2008 The European Chemicals Agency identified HBCDD as 1 of 14 substances of "Very High Concern", and in September 2010 HBCDD was added to REACH's Authorization

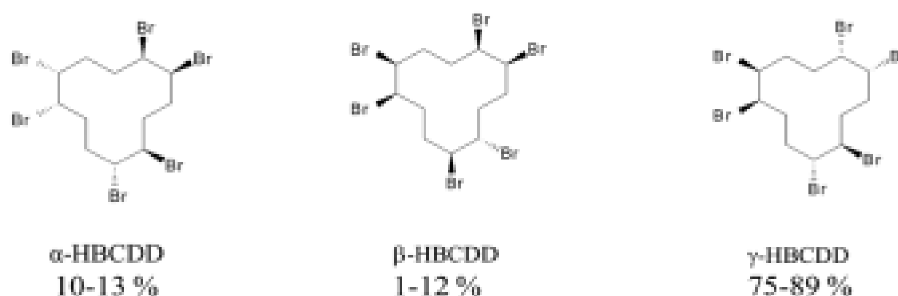
List. In February 2011 HBCDD was selected by EU REACH Regulation to be phased out before 2015, if authorization is not granted to eventual applicants (ECHA 2011).

In May 2013 the Stockholm Convention concluded that HBCDD fulfills the criteria of a persistent organic pollutant (POP) and decided a global ban of HBCDD use with exemptions (Stockholm Convention 2013).

This presentation will discuss the risks associated with HBCDD's prevalence in our homes and immediate environment.

2. Materials/methods

Commercial hexabromocyclododecane (HBCDD) products with CAS no.'s 25637-99-4 or 3194-55-6 are a racemic mixture of mainly three isomers: (i) á-HBCDD; (ii) â-HBCDD and (iii) ã-HBCDD. The structure and approximate composition are shown in **Figure 1:**



The single isomers have slightly different physicochemical characteristics: á-HBCDD is the most thermodynamically stable isomer.

The HBCDD mixture is characterized by very poor water solubility (66 ìg/L at 20°C), high lipophilicity (Log K_{ow}: 5.6), low volatility ($6x10^{-5}$ Pa at 21°C) and a melting point of 190°C.

HBCDD has been increasingly used as a substitute for other brominated flame retardants such as polybrominated diphenyl ethers (PBDEs), and the world production has increased from 16 000 tonnes in 2000 to 23 000 tonnes in 2008, and a great part of this increase has occurred in China (Klif 2011).

Almost 80% of HBCDD produced is estimated to be used as a flame retardant in expanded polystyrene (EPS) and extruded polystyrene (XPS) insulation products for buildings and construction. In addition, HBCDD is applied to high impact polystyrene in electrical or electronic parts. It can also be found in polymer-dispersion coating agents used in textiles for upholstered fabric, furniture, mattress ticking and for seating in vehicles (EU 2008).

3. Results

It is estimated that the EPS/XPS market share includes about 35% of the building insulation in Europe. It is a relatively cheap and energy-efficient material easy to work with. There are many producers in Eastern Europe, and these are often SMEs. Because this material consists of 90% air which result in a relatively large volume making only a short distribution distance economically feasible.

In the EU Risk Assessment from 2008 (EU 2008) emissions of HBCDD into Europe were estimated (see Table 1). It showed that air emissions came mainly from industrial use and

 ISBN: 978-81-928924-3-6

installation of boards, and emissions into waters mainly originate from the textile industry. However, this release assessment was incomplete, since emissions into the terrestrial environment and indoors was not included. The later life cycle stages, waste and recycling, were neither included. Buildings may have a lifespan of more than 50 years but at demolition the remaining HBCDD will be emitted to the environment.

Furthermore, the use of HBCDD as a substitute for PBDEs was not considered. Recent studies of an E-waste facility in China showed that this facility had polluted the surroundings with 1.8 tonnes of HBCDD, and there were increased HBCDD-levels in the soil at a distance of 50 km from the plant (Gao *et al.* 2011).

Trend studies of HBCDD in lake sediments have shown increasing levels since beginning of the 1980s (Kohler *et al.* 2008). In about the same period background levels of HBCDD (mainly the a-isomer) have also increased in wildlife (Vorkamp *et al.* 2011; Johansson *et al.*2011). In areas with high levels because of industrial point sources levels have decreased after closure of plants (Law *et al.* 2008).

Human exposures are mainly at work places or through food intakes, however, exposure to indoor air and dusts may sometime be more important (Roosens *et al.* 2009). Vacuum cleaner dusts in the U.K. contained until 110 ppm HBCDD as showed in Figure 2.

Table 1: HBCDD Emissions in Europe (from EU 2008):

Life-cycle stage	**Total HBCDD emissions in Europe (kg/year)**		
	Air	**Waste water**	**Surface water**
Production	2.0	0.7	0
Micronizing	0.3	0	0
Formulation of EPS and HIPS	20	48	**212**
Formulation of XPS	11	71	8.5
Formulation polymer dispersion	6.8	**220**	55
Industrial use EPS	**102**	82	20
Industrial use HIPS	6.3	5.0	1.3
Industrial use XPS (compound)	**100**	27	7
Industrial use XPS (powder)	24	26	6.6
Industrial use textile back coating	0.6	**5653**	**1413**
Insulation boards, professional use	**182**	0	182
Service life textiles (washing)	0	10	0
Service life textiles (wear)	0	107	27
Service life EPS/XPS	54	0	0
Total emissions	**508**	**6251**	**1933**

Table 2: HBCDD (ìg/kg) in vacuum cleaner dusts from different countries (Abdallah *et al.* 2008).

Place	No. samples	Range	Mean	Median	Distribution of isomers (%)		
			ìg/kg		á	b	g
Birmingham, UK, home	31	140 -110000	6000	730	32	8	60
Birmingham, UK, office	6	90 – 3600	1400	650	21	10	70
UK office and homes	10	940 - 6900	3160	-	-	-	-
Belgian offices and homes	23	<20 - 58000	4800	-	-	-	-
Offices in the EU	18	<3 – 3700	-	-	-	-	-
US homes	17	<3 – 925	140	-	-	-	-
Austin, USA	13	110 - 4000	810	390	28	7	65
Toronto, Canada	8	64 -1300	670	640	49	10	41

HBCDD is absorbed into the body through the respiratory and gastro intestinal tracts and accumulates in body fats. Levels in humans are often monitored by analyzing samples of blood or human milk. HBCDD concentrations in pooled mothers' milk samples from a milk bank in Stockholm, Sweden, show an increasing trend over a 30-year period as illustrated in Figure 2:

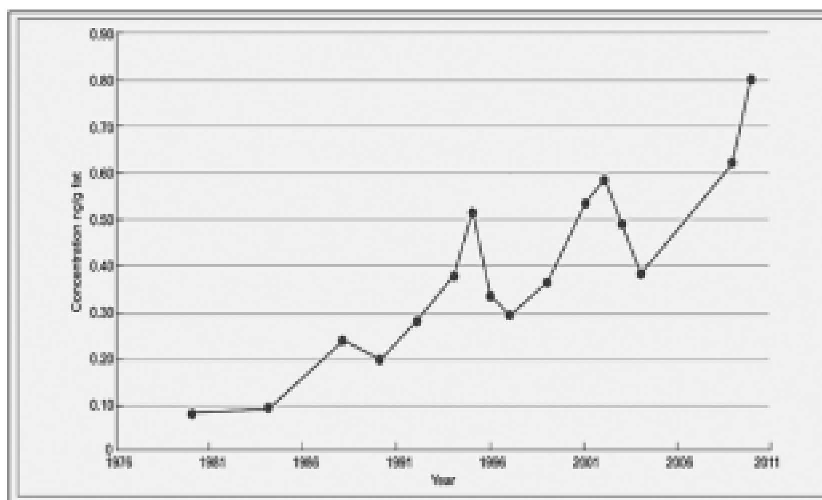


Figure 2: Time trend of HBCDD concentration in Swedish (Stockholm) mothers' milk (Fängström et al. 2008, EFSA 2011).

 ISBN: 978-81-928924-3-6

The levels of HBCDD in human milk from Sweden were low compared to higher average levels of 5-19, 4.3 (max 78), 2.2 (max 13) ng/g fats in Australia, China (Beijing) and a dump-site in India (Kolkata), respectively (Toms et al. 2012¸Shi et al. 2013¸ Devanathan et al. 2012).

HBCDD has a very low acute toxicity in animals by oral and dermal routes of administration. Mainly it targets biotransformation processes in the liver with induction of CYPs and phase II enzymes involved with drug, steroid and thyroid hormones metabolism.

The most consistent effect found after repeated doses in rats was a dose-dependent increase in liver weight. A no-observable-adverse-effect-level (NOAEL) of 22.9 mg/kg per day was estimated for this effect in female rats. Pituitary and thyroid weight also increased significantly, accompanied by thyroid follicular cell hypertrophy. This effect on the pituitary induces increased the synthesis of thyroid-stimulating hormone (TSH).

Exposure to HBCDD can have wide-ranging and potentially severe effects – particularly to the neuroendocrine system and to offspring during the early phases of development. In a two-generation study on HBCCD’s impact on reproduction, thyroid effects were observed both in dams and offspring (Ema *et al.* 2008). The NOAEL in that study was estimated to 10 mg/kg per day.

Another study indicated that neonatal HBCDD exposure may cause statistically significant changes in spontaneous behavior, and learning and may also induce memory defects. An indicative lowest-observable-adverse-effect-level (LOAEL) of 0.9 mg/kg per day was also deduced from this study (Eriksson *et al.* 2006).

In 2011 the European Food Safety Agency EFSA has published a Scientific Opinion on Hexabromocyclododecanes (HBCDDs) in Food (EFSA 2011). It concluded that due to the limitations and uncertainties in the database the derivation of health based guidance values for HBCDDs was not appropriate.

HBCDD was included in the ‘San Antonio Statement on Brominated and Chlorinated Flame Retardants’ signed in September 2010 by 245 scientists from 22 countries (DiGangi *et al.* 2010).

4. Discussion and conclusions

HBCDD is known to be lipophilic, persistent and to accumulate extensively and increasingly in the food chain and in humans and it is an endocrine disruptor in animals.

However, only limited information is available on its toxicology and nothing is known about the differences in susceptibility between animals and humans or interactions with other chemicals (cocktail effects).

As a result, it is currently not possible to provide a reliable assessment of the long-term implications of exposure to this substance to human health and the environment.

Despite this, HBCDD continues to be used extensively even though:

- Flammable insulation materials made from renewable sources and with less dangerous flame retardants exist.
- Non-flammable insulation alternatives are available – such as mineral wool (stone wool and glass wool), foam glass, aerogel and perlite which do not require the use of flame retardants.

- Safer chemical flame retardant alternatives to HBCDD for EPS/XPS may also be available (Klif 2011, USEPA 2013).

Thus, the prioritizations by ECHA and the Stockholm Convention were correct and timely!

5. Acknowledgement

The research on HBCDD has been supported financially by Rockwool International A/S.

References

1. Abdallah M.A.-E., Harrad S. and Covaci A.. Hexabromocyclododecanes and tetrabromobisphenol-A in indoor air and dust in Birmingham, UK: Implication for human exposure. *Environ. Sci. Technol.,*2008, 42, 6855–6861.
2. Devanathan G., Subramanian A., Sudaryanto A., Takahashi S., Isobe T. and Tanabe S. Brominated flame retardants and polychlorinated biphenyls in human breast milk from several locations in India: Potential contaminant sources in a municipal dumping site. *Environ.Inter.*, 2012, 39, 87-95.
3. DiGangi J., Blum A., Bergman Å., de Wit C.A., Lucas D., Mortimer D., Schecter A., Scheringer M., Shaw SD. and Webster T.F. Editorial: San Antonio Statement on Brominated and Chlorinated Flame Retardants. *Environ. Health Perspect.,*2010,118, A516–A518.
4. ECHA. 2011. http://ECHA.europe.eu/.
5. EFSA. *Scientific Opinion on Hexabromocyclododecanes (HBCDDs) in Food*. EFSA Journal, 2011.,9 (7), 2296.
6. Ema M., Fujii S., Hirata-Koizumi, M. and Matsumoto, M. Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats. *Reprod Toxicol*, 2008, 25, 335–351.
7. Eriksson P., Fisher C., Wallin M., Jakobsson E. and Fredriksson A.. Impaired behaviour, learning and memory, in adult mice neonatally exposed to hexabromocyclododecane (HBCDD). *Environ. Toxicol. Pharmacol.*,2006, 21, 317–322.
8. EUROPEAN UNION (EU). 2008. *Risk Assessment Report on Hexabromocyclododecane, CAS-No. 25637-99-4*. Final draft May 2008. http://ecb.jrc.ec.europa.eu/.
9. Fängström B., Athanassiadis I., Odsjö T., Norén K. and Bergman Å.. Temporal trends of polybrominated diphenyl ethers and hexabromocyclododecane in milk from Stockholm mothers, 1980-2004. *Mol. Nutr. Food Res.*,2008, 52, 187–193.
10. Gao S., Wang J., Yu Z., Guo Q., Sheng G. and Fu J..Hexabromocyclododecanes in surface soils from E-waste recycling areas and industrial areas in South China: concentrations, diastereoisomer- and enantiomer-specific profiles, and inventory**.***Environ. Sci. Technol.,*2011,45, 2093–2099.
11. Johansson A.-K., Sellström U., Lindberg P., Bignert A. and De Witt C.A.. Temporal trends of polybrominated diphenyl ethers and hexabromocyclododecane in Swedish Perigrine Falcon (*Falco perigrinus perigrinus*) eggs. *Environ. Int.*,2011, 37, 678-686.
12. Klif. 2011. *Alternatives to the use of flame retarded EPS*. TA-2827. http://www.klif.no/no/Publikasjoner/Publikasjoner/2011/Juli/Alternatives-to-the-use-of-flame-retarded-EPS/.
13. Kohler M., Zennegg M., Bogdal C., Gerecke A.C., Schmid P., Heeb N.V., Sturm M., , Vonmont H., Kohler H-P.E. and Giger W.. Temporal Trends, congener patterns, and sources of octa-, nona-, and decabromodiphenyl ethers (PBDE) and hexabromocyclododecanes (HBCD) in Swiss Lake Sediments. *Environ. Sci. Technol.*,2008, 42, 6378–6384.
14. Law R.J., Bersuder P., Brady J., Wilford B.H., Allchin C.R. and Jepson P.D.. A significant downturn in levels of hexabromocyclododecane in the blubber of Harbour Porpoise (*Phocoena*

phocoena) stranded or caught in the UK: an update to 2006. *Environ. Sci. Technol.*,2008, 42, 9104–9109.

15. NORDIC COUNCIL OF MINISTERS. 2008. *Hexabromocyclododecane as a possible global POP***.**TemaNord. Copenhagen, Denmark.
16. Roosens L., Abdallah M.A.-E., Harrad S., Neels H. and Covaci A.. Exposure to Hexabromocyclododecanes (HBCDs) via Dust Ingestion, but Not Diet, Correlates with Concentrations in Human Serum: Preliminary Results. *Environ. Health Perspect*.,2009, 117, 1707-1712.
17. Shi Z., Jiao Y., Hu Y., Sun Z., Zhou X., Feng J., Li J. and Wu Y.. Levels of tetrabromobisphenol A, hexabromocyclododecanes and polybrominated diphenyl ethers in human milk from the general population in Beijing, China. *Science Total Environ.,*2013,452–453, 10–18.
18. Stockholm Convention. 2013. http://chm.pops.int/
19. Toms L.-M.L., Guerra P., Eljarrat E., Barceló D., Harden F.A., Hobson P., Sjodin A., Ryan E. and Mueller J.F.. Brominated flame retardants in the Australian population: 1993–2009. *Chemosphere*,2012, 89, 398–403.
20. USEPA . 2013. *Flame retardant alternatives for hexabromocyclododecane (HBCD).* Draft for public comment, September 2013.
21. Vorkamp K., Rigét F.F., Bossi R. and Dietz R.. Temporal trends of hexabromocyclododecane, polybrominated diphenyl ethers and polychlorinated biphenyls in ringed seals from East Greenland. *Environ. Sci. Technol.*,2011, 45, 1243-1249.

Chapter 3

Turning beauty into the beast: pharmaceuticals and personal care products in the aquatic environment

***Olga I. Kalantzi[1], Panayotis A. Siskos[2]**
[1] Department of the Environment, University of the Aegean, 81100 Mytilene, Greece
[2] Department of Chemistry, National and Kapodistrian University of Athens, Panepistimioupoli, 157 71 Zografos, Athens, Greece
***Corresponding author:** kalantzi@aegean.gr

Abstract

Pharmaceuticals and personal care products (PPCPs) are a very diverse group of thousands of chemicals substances, such as prescription and over-the-counter therapeutic drugs, fragrances, shampoos, cosmetics, sunscreen agents, diagnostic agents, biopharmaceuticals, and many other everyday anthropogenic products. The main sources of PPCPs in the environment are human activity, residues from pharmaceutical manufacturing and hospitals, illicit drugs, veterinary drug use (especially antibiotics and steroids) and agribusiness (US EPA, 2010). PPCPs have been found in water bodies all around the world, including rivers, streams, ground water, coastal marine environments and drinking water sources (Daughton, 2004).

Very few monitoring systems currently exist to regulate these chemicals, because most of them focus on the direct effects of these substances on humans, rather than after-use effects on the environment. As large volumes of these human wastes enter the water supply on a daily basis, the concentrations of the chemicals they comprise of continue to increase, as do their potential toxic effects.There are a number of reasons why PPCPs may pose a threat to the environment. The quantities entering the environment have been steadily increasing over the years, there are no municipal sewage treatment plants that target PPCPs and the risks to humans and wildlife are largely unknown (Daughton, 2004). PPCPs are usually present in very low concentrations in the environment, but their activity and behavior in mixtures is may be unpredictable.

Some of the known effects of PPCPs in organisms include delayed development in fish and frogs and a variety of other reactions in organisms including altered behavior and reproduction (Nash *et al.*, 2004).Several fish studies have indicated that exposure to PPCPs that act as synthetic estrogens causes reproductive effects such as changes in sperm density, gonad size, reduced viability of eggs, and male sex reversal (Knörr and Braunbeck, 2000). There are no known human health effects from such low-level exposures in drinking water; however more research is needed to address mixture toxicity and biochemical reactions in

organisms. This paper presents a review of the recent literature in the field, with emphasis on the European environment and highlights the need for monitoring the concentrations of these chemicals in the water supply.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are a group of chemicals, including drugs, cosmetics, fragances and biopharmaceuticals that have been used by humans for decades. Although their existence was widely known for a long time, it has only been about 15 years since they have emerged as major contaminants in the environment. PPCPs have been detected in different parts of the environment, including surface and ground waters, soil, and biota (Daughton and Ruhoy, 2009). The main route of entry into the environment is excretion to sewers through wastewater treatment plants and into the water supply. As large volumes of these human wastes enter the water supply on a daily basis, the concentrations of the chemicals they comprise of continue to increase, as do their potential toxic effects.

Some of the known effects of PPCPs in organisms include delayed development in fish and frogs and a variety of other reactions in organisms including altered behaviour and reproduction (Nash et al., 2004).Several fish studies have indicated that exposure to PPCPs that act as synthetic estrogens causes reproductive effects such as changes in sperm density, gonad size, reduced viability of eggs, and male sex reversal Knörr and Braunbeck, 2000). Although PPCPs have been largely ignored by policy makers, the European Union recently added the anti-inflammatory drug diclofenac to its list of Priority Substances, which shoes that interest in their regulatory consideration is starting to emerge (EU, 2012). This paper presents a review of the recent literature on PPCPs in water, with emphasis on the European environment and highlights the need for monitoring the concentrations of these chemicals in the water supply.

2. PPCPs sources and use

It has been suggested that there may be as many as 6 million PPCP substances commercially available worldwide (Daughton, 2004). In combination with the increasing urbanization and associated activities, as well as the increasing pharmaceutical use around the world, one can imagine the concern over the presence of these chemicals in the environment and their possible health effects. Figure 1 illustrates the sources of PPCPs. These include:

- metabolic excretion by individuals (1a) and pets (1b)
- release of hospital wastes to domestic sewage systems (2)
- release to private septic/leach fields (3a) and treated effluent from domestic sewage treatment plants discharged to surface waters (3b)
- transfer of sewage solids to land (e.g., soil amendment/fertilization)(4)
- direct release to open waters via washing/bathing/swimming (5)
- discharge of regulated/controlled industrial manufacturing waste streams and release from illicit drug usage (6)
- disposal to landfills via domestic refuse, medical wastes, and other hazardous wastes and leaching from defective landfills (7)
- release to open waters from aquaculture (medicated feed and resulting excreta)(8)

- release of drugs that serve as pest control agents (9) and
- environmental transport/fate, including phototransformation, physicochemical alteration, volatilization and uptake by plants (10).

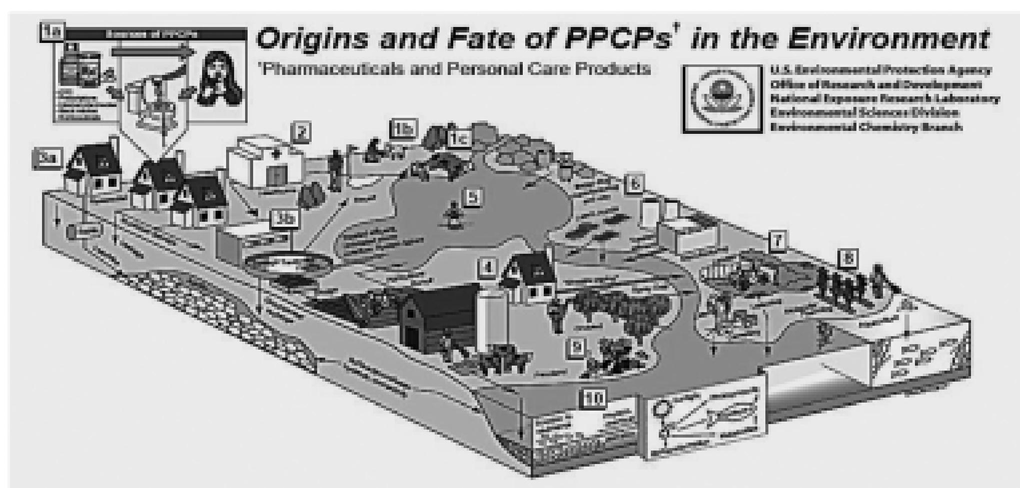


Fig.1:Origins and fate of PPCPs in the environment

It is estimated that Germany uses over 600 tonnes per year of antibiotics, whilst France, Italy and Spain use about 300 tonnes per year. In the UK over 3000 active substances are licensed for use, with paracetamol (2000 tonnes per year) and acetylsalicylic acid (770 tonnes perr year) being the highest usage drugs. A total of 170 pharmaceutical chemicals are estimated to be used in excess of 1 tonne per year in Europe (Daughton, 2004).

3. Levels in European waters

Although it has been known for more than two decades that PPCPs can enter the environment, only in the last decade or so have the analytical methods become sensitive enough to quantify their presence in trace quantities in environmental samples (Garic, 2013). The analytical methods that are usually employed to detect these chemicals in water samples are gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS–MS) and HPLC–electrospray ionization (ESI) MS (Cahill et al., 2004). Tandem mass spectrometry has been especially important in improving knowledge in recent years (Kümmerer, 2009).

Sewage treatment plants (STPs) are the main point discharge sources of these chemicals in the environment. A monitoring campaign of STP effluents carried out in four European countries (Italy, France, Greece and Sweden) detected more than 20 individual pharmaceuticals belonging to different therapeutic classes, in concentrations ranging from 0.01-7.11 ìg/l (Andreozzi *et al.,* 2003). Some pharmaceuticals (such as antibiotics, ibuprofen, diclofenac, carbamazepine and the majority of â-blockers) were detected in almost every sample, as a result of their high prescription extent and wide usage in Europe.

The fate of PPCPs irrigated on arable land with treated municipal wastewater was investigated by Ternes *et al*. (2007) in Germany. The authors monitored six wells and four lysimeters located in one of the irrigated agricultural fields for presence of 52 pharmaceuticals and two personal care products. PPCPs were not detected in any of the lysimeter or groundwater samples, although they were present in the treated wastewater irrigated onto the fields. The authors concluded that potential estrogenic effects are likely to disappear after irrigation, since the most potent steroid estrogens were not measurable.

In the Somes River in Romania 15 PPCPs (including pharmaceutics, metabolites, intermediates and musk fragrances) were determined in concentrations ranging from 30 ng/l to 10 ìg/l (Moldovan, 2006). The determined compounds were arranged in three quantitative groups as a function of their measured concentrations: group I (between 300 and 10 000 ng/ l: caffeine, acetylaminophenazone), group II (between 100 and 300 ng/l: pentaoxifilline, ibuprofen, formylaminophenazone, galaxolide, tonalide, *p*-chlorophenyl sulfone, *N*,*N*-bis(3,3-dimethyl-2-oxetanyl)-3,3-dimethyl-2-oxetan-amine) and group III (under 100 ng/l: aspirin, triclosan, carbamazepine, codeine, diazepam and cyclofosfamide).

The presence of 6 pharmaceuticals and personal care products was investigated in drinking water from the Llobregat river basin in Spain. Waters from the outlet of various sewage treatment plants (STP) and waterworks located along the river basin, as well as water samples from the river or its tributaries upstream and downstream of these plants were analysed. Except for one, all PPCPs studied (ibuprofen, diclofenac, clofibric acid, salicylic acid, and triclosan) were determined at levels usually lower than150 ng/l and up to 1200 ng/l (in the case of diclofenac) (Kuster *et al*., 2008).

A large pan-European study in 23 European countries reported that the most relevant chemicals found for ground water infiltration and pollution were caffeine and carbamazepine (Loos *et al*., 2010). Compared to river surface water, ground water was in general less contaminated, with an average frequency of detection for all compounds of 25%.

There have been some studies trying to assess and improve the PPCP removal efficiency of drinking water technologies. According to the EU POSEIDON project report (EU Poseidon Project, 2007), biological degradation and sorption are the main mechanisms for PPCP removal during municipal wastewater treatment. Ozonation and activated carbon have been tested for the removal of PPCPs from water (Sánchez-Polo*et al*., 2008). Nanofiltration and ultrafiltration membranes have been suggested as a removal tool for PPCPs from drinking water sources (Yoon *et al*., 2007). More recently liquid core microcapsules have been suggested as a novel way to extract PPCPs from aqueous solutions (Whelehan *et al*., 2010). This method has the advantage of more efficient extractions and reusability of the capsules, which makes it an overall more economical option.

4. Effects in organisms

The effects of PPCPs are many and varied, depending on the individual chemical. Environmental toxicology focuses on acute effects of exposure rather than chronic effects. It is important to remember that these effects may be subtle because PPCPs occur in the environment at very low concentrations, however this doesn't make them less significant.

The redox activity of various PPCPs in trout (*Oncorhynchus mykiss*) liver microsomes was investigated *in vitro* by Gagne *et al*. (2006). Results showed that most PPCPs (83%) accelerated the rate of NADPH oxidation in the presence of microsomes. Exposure of trout hepatocytes to these products leads in many cases to decreased cell viability, increased CYP3A-related monooxygenase activity and LPO. Municipal effluent extracts were able to increase all the above responses in a dose-dependent manner, which suggests that the basic redox properties of PPCPs could influence oxidative metabolism in liver cells and lead to oxidative damage.

Male fish *G. holbrooki* exhibited a highly responsive abnormal behaviour following exposure to diazepam (Nunes *et al*., 2008). The individual and mixture toxicity of six PPCPs to the marine phytoplankton species *Dunaliella tertiolecta* using a standard 96-hour static algal bioassay protocol were investigated by DeLorenzo and Fleming (2008). All PPCPs tested had a significant effect on *D. tertiolecta* population cell density. Both mixtures tested demonstrated additive toxicity, which may decrease the toxicity threshold for phytoplankton populations. Detrimental effects on phytoplankton populations could ultimately impact nutrient cycling and food availability to higher trophic levels.

Atorvastatin, carbamazepine, 17á-ethinylestradiol and triclosan were found to be toxic only at concentrations at least 100-fold greater than those typically detected in aquatic environments in two types of benthic invertebrates (the midge *Chironomus tentans* and the freshwater amphipod *Hyalella azteca)(Dussalt et al., 2008).*

Acute toxicity testing approaches may be inappropriate to evaluate the long-term chronic potency of PPCPs, as they ignore potential persistence, liability to continued low-dose exposure and chronic long-term bioaccumulation potential, especially by uptake from contaminated benthic sediment in urban receiving waters.

References

1. Andreozzi, R., Raffaele, M., Nicklas, P. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment, *Chemosphere*, 2003, 50 (10), pp. 1319–1330.
2. Cahill, J.D., Furlong, E.T., Burkhardt, M.R., Kolpin, D., Anderson, L.G. . Determination of pharmaceutical compounds in surface- and ground-water samples by solid-phase extraction and HPLC-electrospray ionisation MS, *J. Chromatogr. A,* 2004,1041, pp. 171–180.
3. Daughton, C.G. Non-regulated water contaminants: emerging research. *Environmental Impact Assessment Review*,2004, 24, pp. 711–732.
4. Daughton, C.G. and Ruhoy, I.S. Environmental footprint of pharmaceuticals: the significance of factors beyond direct excretion to sewers. *Environmental Toxicology and Chemistry*, 2009, 28 (12), pp. 2495-2521.
5. DeLorenzo, M.E., Fleming, J..Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species Dunaliella tertiolecta.*Archives of environmental Contamination and Toxicology*2008, 54 (2), pp. 203-210.
6. Dussault, E.B., Balakrishnan, V.K., Sverko, E., Solomon, K.R. and Sibley, P.K. . Toxicity of human pharmaceuticals and personal care products to benthic invertebrates. *Environmental Toxicology and Chemistry.*2008, 27 (2), pp. 425-32.
7. EU, 2012. Environment and Water: proposal to reduce water pollution risks. http://europa.eu/rapid/press-release_IP-12-88_en.htm [last accessed 19/11/13]

8. EU Poseidon Project. (2007). Assessment of technologies for the removal of pharmaceuticals and personal care products in sewage and drinking water facilities to improve the indirect potable water reuse.http://poseidon.bafg.de/servlet/is/2888/ [last accessed: 19/11/2013].
9. Gagné F, Blaise C, André C..Occurrence of pharmaceutical products in a municipal effluent and toxicity to rainbow trout (Oncorhynchus mykiss) hepatocytes.*Ecotoxicol. Environ. Saf.* 2006, 64 (3), pp. 329-36.
10. Garric, J. Emerging Issues in Ecotoxicology: Pharmaceuticals and Personal Care Products (PPCPs). *In:* Ferard, J.-F. and Blaise, C. (eds.) *Encyclopedia of Aquatic Ecotoxicology.* Springer Netherlands.2013.
11. Knörr, S. Braunbeck, T.. Decline in reproductive success, sex reversal, and developmental: alterations in Japanese medaka (*Oryzias latipes*) after continuous exposure to octylphenol, *Ecotoxicol. Environ. Safe.*,2002, 51, pp. 187–196.
12. Kümmerer, K..The presence of pharmaceuticals in the environment due to human use - Present knowledge and future challenges.*Journal of Environmental Management*, 2009, 90 (8), pp. 2354–2366.
13. Kuster, M., Lopez de Alda, M.J., Hernando, M.D., Petrovic, M., Martin-Alonso, J., Barcelo, D. Analysis and occurrence of pharmaceuticals, estrogens, progestogens and polar pesticides in sewage treatment plant effluents, river water and drinking water in the Llobregat river basin (Barcelona, Spain). *Journal of Hydrology*,2008, 358 (1), pp. 112-123.
14. Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., Manfred, Gawlik, B. Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. *Water Research*, 2010, 44 (14), pp. 4115-4126.
15. Moldovan, Z. Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania. *Chemosphere*,2006, 64 (11), pp. 1808-1817.
16. Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish, *Environ. Health Perspect.*,2004, 112, pp. 1725–1733.
17. Nunes, B., Gaio, A.R., Carvalho, F., Guilhermino, L. Behaviour and biomarkers of oxidative stress in *Gambusia holbrooki* after acute exposure to widely used pharmaceuticals and a detergent. *Ecotoxicology and Environmental Safety*,2008, 71 (2), pp. 341-354.
18. Sánchez-Polo, M., Rivera-Utrilla, J., Prados-Joya, G., Ferro-Garcia, M.a., Butista-Toledo, I. Removal of pharmaceutical compounds, nitroimidazoles, from waters by using the ozone/carbon system.*Water Research*,2008, 42 (15), pp. 4163-4171.
19. Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. *Chemosphere*,2007, 66 (5), pp. 894-904.
20. US Environmental Protection Agency (US EPA). Pharmaceuticals and personal care products (PPCPs). 2010, http://www.epa.gov/ppcp/faq.html [last accessed: 19/11/13]
21. Whelehan, M., von Stockar, U. and Marison, I.W. Removal of pharmaceuticals from water: Using liquid-core microcapsules as a novel approach. *Water Research*, 2010, 44 (7), pp. 2314-2324.
22. Yoon, Y., Westerhoff, P., Snyder, S.A., Wert, E.C., Yoon, J.Removal of endocrine disrupting compounds and pharmaceuticals by nanofiltration and ultrafiltration membranes.*Desalination*,2007, 202 (1-3), pp. 16-23.

Chapter 4

Epigenetic Response to Environmental Pollutants and its Effects on Human Health: A Review

Spencer Keene, Paul McFadden and *S.K. Majumdar
Kreider Emeritus Professor of Biology,
Former President of the National Association of Academies of Science,
The Pennsylvania Academy of Science, USA
Department of Biology, Lafayette College, Easton, PA. USA, 18040
***Corresponding author:** majumdas@lafayette.edu

Abstract

Environmental chemicals and lifestyle choices have long played a role in human health. Many environmental chemicals and certain lifestyle factors are known to damage DNA and altering gene's normal activity. In recent years, scientists have reported changes in gene functions resulted from DNA methylation and histone modifications, without changing the DNA base pair sequence. This emerging field of study is called epigenetics. Although epigenetic events do not alter the DNA sequence, some of which are heritable, however. The epigenetic modifications can be brought about by diet, lifestyle choices and environmental factors, resulting in the alteration of gene expression. DNA methylation occurs when methyl groups (epigenetic factors) from environmental pollutants and dietary sources attach to one of the DNA bases (mainly cytosine) resulting in epigenetic misregulations. These changes can abnormally switch genes on or off. For example, altered DNA methylation can silence a tumor suppressor gene and activate an oncogene leading to the development of cancer. The aberrant epigenetic signals are reported to increase the risk of developing cancer, age related diseases and other metabolic ailments such as diabetes and cardiovascular abnormalities. Epigenetic changes are also linked to abnormal zygote development and possibly stem cell differentiation. Histone modifications result from irregular acetylation and methylation of histone proteins can also contribute to human diseases. These epigenetic alterations change the DNA's wrapping capability around histone proteins and derange the on-off switches that control the expression of specific genes. Based on laboratory animal experiments, misregulated epigenetic events in sperm and eggs are found to be transgenerational. Since epigenetic changes can be identified earlier than the onset of a disease, it is suggested that this phenomenon can have some potential prognostic value. Additionally, epigenetic alterations in the promoter region of micro-RNAs have been shown to inhibit tumor cell growth. In this review I will discuss roles of several

environmental chemicals and certain life style factors in the production of aberrant epigenetic signals and their consequences in developing certain human diseases.

1. Introduction

Environmental chemicals and diet factors have long played a role in human health. Many environmental pollutants act as mutagens and induce mutations by damaging DNA bases leading to abnormal DNA replication, transcription and translation. Genetic materials of eukaryotic organisms reside in the chromosomes. Chromosomes are made up of tightly coiled chromatin fibers. The main components of chromatin fibers include a polynucleotide DNA chain, repeating units of four pairs of histone core proteins, DNA binding proteins and some RNA. A total of ~146base pairs of DNA wraps around each octamer of histones forming a structure called a nucleosome. A fifth histonecalled H1 is located in the short "linker" region of DNA between nucleosomes making the chromatin fiber more condensed and compact. Chemical modifications of nucleosomes determine which genes will be unwound and accessible for further processing and which ones will remain inaccessible. Amino acid "tails" project out from each nucleosome. The Cytosine-Guanine dinucleotide sites on the DNA chain and the N- terminal of histone "tails" are the preferred binding sites for epigenetic factors.

Genetic mutations are generally induced by environmental genotoxins and some of them are carcinogenic or pre-carcinogenic. Besides the genotoxin-induced mutations, certain percentages of mutations arise spontaneously and are genetically predisposed. Mutations can be induced not only by natural and man-made chemicals found in the environment, but also by occupational exposure in the workplace. Mutations can lead to various diseases including cancer. When mutations originate in germ cells, they have the capacity of transmitting the condition to the next generation. Irrespective of whether a mutation is produced in somatic or germ cells, it has the potential of causing illness by disrupting the structure and function of proteins. Different mutations in the same or different genes may cause distinct illnesses. Similarly, diseases like diabetes mellitus, cancers and many other diseases can be produced by different genes or by changing their normal epigenetic states (Stein, 2014). Alterations to theepigenome induced by epigenetic factors are the main topic of this review paper and is presented in the following discussion.

Arelatively new area in the genetic field has emerged as epigenetics. Epigenetic refers to the study of changes in gene regulation and expression leading to non-heritable and heritable conditions independent of an alternation in the DNA sequence. C.H. Waddington first introduced the term in 1942, but it did not catch the attention of scientists immediately. This lack of interest, however, has turned around in recent years after realizing its impact on biomedical fields. Recent studies have shown that many environmental toxicants,including factors from dietary sources**,**can induce epigenetic modifications leading to alterations in gene functions (Fragou, et al. 2011; Kubicek, 2011; Abrendt, 2009; Zeisel, 2009; Shukla, 2008). Epigenetic phenomenon in organisms is well balanced. Disruptions of this epigenomic balance by environmental chemicals can, in many cases, trigger the development ofdiseases, including cancer. For example, DNA or genomic imprinting is an example of epigenetic

phenomenon where only one copy of genes is expressed; parental origin influences the phenotype. The turning on or off of the gene (s) is controlled by the parent's set patterns of DNA methylation and histone modifications. Any alterations in these arrangements would change the set functions of the genes and prevent them from being accessed fortranscription and translation leading to phenotypic and genotypic changes in the organisms. Epigenetic variations are caused by changes in DNA methylation/demethylation, histone modifications as well as MicroRNAs alterations. This review will focus on how the epigenome responds to environmental contaminants and how the resultant responses affect human health.

2. DNA Methylation

DNA methylation involves the addition of a methyl group to either the cytosine or adenine nucleotides of DNA and has significant roles in gene regulation and expression, as well as maintaining gene stability (Kubicek, 2011; Hou, et al. 2011; Chiang, et al. 1996). Hypermethylation of cytosine usually occurs at cytosine-guanine dinucleotide sites (CpG islands) and "CpG" island-shore, located at the promoter site of the gene (Kubicek, 2011; Jones and Baylin, 2002). The epigenetic modifications disrupt transcription through alteration of the wrapping pattern of DNA strand around the nucleosome leading to gene inactivation (Esteller, 2013). DNA methylation affects male fertility, X-chromosome inactivation, and causes cancer, cardiovascular diseases as well as Rett syndrome, and Prader-Willi/Angelman syndrome (Esteller, 2011; Anway and Skinner, 2013; Lund, 2004). DNA hypo- or demethylation, which occurs mainly in the repetitive DNA sequence**,** activates normally silenced genes. Global hypomethylation increases mutation rate, and is linked to tumorigenesis and normal DNA imprinting processes (Pogribny and Rusyn, 2013). Studies reveal that many epigenetic events are reversible and they can be detected easily utilizing the modern biotechnique assays.

3. Histone and micro-RNA modifications

The DNA is packed tightly in the nucleus in association with histones and other regulatory proteins to form the first level of chromatin structure, called the nucleosome. Each nucleosome is made up of four pairs of core histones (H2A, H2B, H3 and H4) along with ~146 tightly wrapping DNA base pairs. The core histones are characterized by having N-terminal tails, which are the binding sites for epigenetic factors. Histone modifications are mediated by acetylation, deacetylation, methylation, and phosphorylation. Usually, both acetylation and phosphorylation loosen while methylation tightens the histone-DNA interaction and, as a result**,** the transcription factors necessary for transcription initiation are blocked or uninhibited from accessing the DNA(Zeisel, 2009). The various combinations of modifications on histone tails create a "histone code" that regulates chromosome condensation, transcriptional activity, silencing repetitive DNA elements and DNA repair (Rutten and Mill, 2009; Peterson and Laniel 2004). There is overwhelming evidence to link histone modifications to cancer induction (Pogribny and Rusyn, 2013; Esteller, 2013).

MicroRNAs (miRNAs) are short 22-24 RNA nucleotide bases found ubiquitously in eukaryotic cells including humans and they function as negative post-transcriptional gene regulators (Esteller 2013; Hou et al. 2011). By binding to complementary sequences on mRNA,

they prevent translation resulting in silencing the expression of specific genes. Epigenetic alterations of miRNA resulted from hypermethylation of promoter regions have been linked to cancer and other conditions like Alzheimer's and cardiovascular diseases (Pogribny and Rusyn, 2013; Esteller, 2013; Hou, et al. 2011; Saito et al. 2009).

4. Selected environmental chemicals and epigenetic changes

Dioxin is a weak mutagen and a strong human carcinogen. Because of methylation at several places on DNA, the agent up-regulates the microR-191 expression leading to dioxin induced-cancer (Elyakim et al. 2010). Dioxin also causes other diseases and developmental defects. Manikkam et al. (2012), studied dioxin's effects on F3 generation in mice after direct exposure of F0 gestation females and observed transgenerational inheritance. Mice exhibited higher incidences of kidney disease, pubertal abnormality and developmental defects and the effects were gender specific.

Dieldrin, a neurotoxin organochlorine pesticide**,** damages dopamine related neuronal cells through hyperacetylation of histone protein (Song et al. 2010; Hou, et al. 2011). Histone acetylation can be reversed somewhat by anacardic acid, a histone acetyltransferase inhibitor (Song et al. 2010).

Pesticideslike**vinclozolin** and **methoxyclor** cause heritable changes resulted from DNA methylation in the testicular germ cells in animal studies (Anway et al. 2005).

Chlorine is used routinely in disinfecting drinking water. The agent produces a number of compounds such as dichloroacetic acid, trichloroacetic acid, chloroform, and trihalomethanes along with other chlorinated compounds in drinking water. Many of these compounds have been implicated to various ailments in humans (Uden and Miller, 1983; Colman, et al. 1984). These agents increase c-myc gene expression due to DNA hypomethylation of the c-myc promoter resulting in liver cancer (Coffin, et al. 2000).

Benzene is widely present in the environment but it is found at higher concentration levels in gas stationsand enclosed auto parking areas. Studies reveal that benzene-induced aberrant epigenetic DNA methylation leads to apoptosis and development of certain kinds of cancer (Bollati et al. 2007; Gao A et al. 2011). Benzene causes decreased methylation of MAGE-1, a gene commonly found expressed in tumor cells and an increase in p15 gene methylation leading to gene suppression; both are associated with acute myeloid lymphoma.

Exposure to **arsenic** can cause a number of diseases including cancers of skin, lung, bladder and liver. Arsenates are less toxic than inorganic arsenite (12- Fragou, et al, 2011; 26- Chanda, et al. 2006). Arsenic exposure induces global hypomethylation and gene specific hypermethylation. Arsenic contaminated drinking water methylates p53 and p16 tumor-suppressor genes. The agent also affects histone methylation via di- and trimethylation of histone H3K9. Mice exposed to arsenic *in utero* experienced higher incidences of cancer in adulthood (McGowan et al. 2009). Zhao et al. (1997) showed that arsenic induced cell transformation resulted in carcinoma due to DNA demethylation.

Mercury is widely present in the environment and a major public health issue. Methyl mercury synthesized from inorganic mercury by anaerobic bacteria in nature, is highly neurotoxic to humans and animals (Poulain and Barkay, 2013). Parks et al. (2013) identified

two genes (*hgcA* and *hgcB*) from anaerobic bacteria that are involved in mercury methylation. Goodrich et al (2013), reports that mercury is associated with DNA hypermethylation of human *SEPP1*, a gene involved in minimizing mercury toxicity, a major concern to human health *e.g.* Minamata disease.

Cadmium, a heavy metal is used widely in industry and its prolonged exposure is linked to liver, lungs, renal and other cancers (Fragou, et al. 2011). Taguchi et al. (2003) showed that cadmium induced-hypomethylation of oncogenes enhanced the cell proliferation and increased the risk of cancer development. Bollati et al., (2010) reported an increased expression of microRNA-146 among steel workers exposed to cadmium-rich environment and its possible association with inflammation and cancer.

Lead is one of the most toxic heavy metals found in the environment. Humans are exposed to the agent through soil, water, air, foods, etc. Lead is linked to many kinds of human diseases, the most prominent one is its neurotoxic effects on the central nervous system, leading to irreversible mental disorders including, learning disabilities (Fragou, et al. 2011; Hou, et al. 2011). Prolonged exposure to leadis reported to change epigenetic marks through DNA methylation. It is suggest that, these epigenetic alterations might be involved in producing some late onset diseases in humans (Fragou, et al. 2011).

Nickel induces cancer through DNA methylation and histone modification. Tumors induced by nickel have hypermethylated tumor suppressor genes p16 (Govindarajan, et al. 2002) and FHIT (Kowara et al. 2004). These alterations cause gene silencing leading to malignant transformation. Such increases in methylation can be attributed to the inhibition of demethyltransferase activity, which can be reversed via exposure to a secondary drug that increases the potency of demethylation activity (Cehn, et al. 2010). Nickel- induced histone deacetylation produces global suppression of gene transcription, which can result in cancer through the silencing of genes that help in the stability and repair of the genome (Fragou,et al. 2011; 92- Kang, et al. 2003).

Humans are exposed to **chromium** via inhalation mainly from catalytic converter emissions and from workplaces involved in the production of stainless steel, pressure treated wood, chrome plating, etc. (Fragou, et al. 2011). Chromium-induced DNA methylation is linked to lung cancer found among factory workers who are exposed to the metal (Kondo, et al. 2003; Takahashi, et al. 2005). Sun et al. (2009) showed that chromium caused changesin histone acetylation resulting in decreased activity of the tumor suppressor gene MLH1. These authors suggested that epigenetic silencing of MLH1 might be an explanation for chromium VI carcinogenicity.

5. Conclusions

Humans have between 20,000- 25,000 genes, but only a few are active because of the “on” or “off” states of the genes. Genes are made up of chromatin fibers, which in turn contain mainly DNA and histone proteins. Modifications of DNA and histone proteins can turn a gene “on” or “off. Numerous studies have shown that environmental pollutants can alter physical structure of DNA and associated histones without changing the DNA base sequence. This phenomenon causes both heritable and non-heritable changes in gene regulation and

expression; the study of these events falls under the discipline, epigenetics. The mechanisms that produce the epigenetic changes are DNA methylation and modifications of histones and microRNA.

The epigenetic variations caused by toxicants can have severe impact on human health leading to cancer and other diseases. The mechanisms of altered epigenetic events are now well understood and the epigenetic variants can be easily identified. It is reported from various studies that epigenetic alterations can serve as markers for preventative measures to avert exposure to toxicants as well as diagnostic and prognostic biomarkers for many epigenetically - induced diseases. This is due to the fact that most epigenetic events are a) easily detectable and appear in greater numbers than genetic events, b) appear earlier than the onset of a disease, c) reversible, d) bio-assays are not expensive and easily available and, e) applicable to both toxic and non-toxic substances (Lifang, et al. 2011; Pogribny and Rusyn, 2013). Because of its applicability in biomedical fields, epigenetics is rapidly becoming an important discipline in biology, medicine, pharmacology, cancer and environmental research.

References

1. Abrendt S, Chow J, Yang S, Wu L, Zhang M, Jen J, and Sidransky D. Alcohol Consumption and Cigarette Smoking Increase the Frequency of p53 Mutation in Non-small Cell Lung Cancer. Cancer Research, 2009,60,3155-3159.
2. Anway, MD. et al. Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility. Science, 2005, 308, 1466-1469.
3. Anway, MD. and Skinner, MK. Epigenetic Transgenerational Actions of Endocrine Disruptors. http://press.endocrine.org/doi/full/10.1210/en.2005-1058 Published online: July 01, 2013.
4. Bollati V. et al. Exposure to Metal-rich Particulate Matter Modifies the Expression of Candidate microRNAs in Blood Peripheral Leukocytes. Environ. Health Prospect, 2010, 118,763-768.
5. Bollati V. et al. Changes in DNA Methylation Patterns in Subjects Exposed to Low-Dose Benzene. Cancer Res. 2007, 67, 876-880.
6. Coffin JC et al. Effect of Trihalomethanes on Cell Proliferation and DNA Methylation in Female B6C3F1 Mouse Liver.Toxico. Sci. 2000, 58, 243-252.
7. Cehn H, Ke Q, Kluz T, Yan Y, Costa M. Molecular Targets of Epigenetic Regulation and Effectors of Environmental Influences. Toxicol Appl Pharmacol , 2010, 245, 378-393.
8. Colman WE. et al. Environ Sci Technol. 1984, 18, 674-678.
9. Chanda S. et al. Toxicological Sciences, 2006, 89(2), 431-437.
10. Chiang PK, Gordon RK, Tal J et al. S-Adenosylmethionine and methylation. FASEB J. 1996, 10, 471-480.
11. Elyakim, E. et al. *has*-miRNA-191 is a Candidate Oncogene Target for Hepatocellular Carcinoma Therapy. Cancer Res. 2010, 70, 8077-8087.
12. Esteller M. Epigenetic Changes in Cancer. The Scientist, March 2013, 34-39.
13. Fragou D, Fragou A, Kouidou S, Njau S, and Kovatsi L. Epigenetic Mechanisms in Metal Toxicity. Toxicology Mechanisms and Methods, 2011,21(4),343-352.
14. Furey, TS. and Sethupathy, P. Genetics Driving Epigenetics. Science, 2013,342, 705-706.
15. Gao A. Epigenetic Modification Involved in Benzene-Induced Apoptosis Through Regulating Apoptosis Related Genes Expression. Cell Biol Int. 2011, 35, 391-396.

16. Goodrich et al. Mercury biomarkers and DNA methylation among Michigan Dental Professionals. Environmental and Molecular Mutagenesis 2013, 54 (3), 195–203.
17. Govindarajan B, Klafter R, Miller MS, Mandur C, Mizesko M, Bai X, LaMontagne K Jr., Arbiser JL. Reactive Oxygen-induced Carcinogenesis Causes Hypermethylation of p16 (Ink4a) and Activation of MAP Kinase. Mol Med, 2002, 8**,**1-8.
18. Hou, et al. Environmental Chemical Exposures and Human Epigenetics. Inter Jour Epidemiol, 2011, 1-27.
19. Jones, PA, and Baylin, SB. The Fundamental Role of Epigenetic Events in Cancer. Nat. Rev. Genet. 2002, 3, 415-428.
20. Kang J, Zhang Y, Chen J, Chen H, Lin C, Wang Q, Ou Y Nickel-induced Histone Hypoacetylation: the Role of Reactive Oxygen Species. Toxicol Sci, 2003, 74, 279-286.
21. Kondo Y, Shen L, Issa JP. Critical Role of Histone Methylationin Tumor Suppressor Gene Silencingin Colorectal Cancer. Mol CellBiol. 2003, 23, 206-215.
22. Kowara R, Salnikow K, Diwan BA, Bare RM, Waalkes MP, Kasprzak KS. Reduced FHIT Protein Expression in Nickel-transformed Mouse Cells and in Nickel-induced Murine Sarcomas. Mol Cell Biochem.2004, 255,195-202.
23. Kubicek S. Epigenetics: A Primer. The Scientist, March 2011, 32-33.
24. Lund, AH.Epigenetics and Cancer.Genes Dev. 2004, 18, 2315-2335.
25. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner, MK. Dioxin (TCDD) Induces Epigenetic Trangenerational Inheritance of Adult Onset Disease and Sperm Epimutations, PLOS 2012,7(9), e46249.
26. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M,Turecki G, Meaney M. Epigenetic Regulation of glucocorticoid Receptorin Human Brain Associates with Childhood Abuse, Nat Neurosci, 2009, 12(3),342-348.
27. Parks, et al.The Genetic Basis for Bacterial Mercury Methylation.Science, 2013, 339, 1332-1335.
28. Peterson, CL. And Laniel MA. Histones and Histone Modifications. Curr.Biol,2004,14(14), R546–R551. (PubMed)
29. Pogribny, IP. and Rusyn, I. Environmental Toxicants, Epigenetics, and Cancer In: Epigenetic Alterations in Oncogenesis, Advances in Experimental Medicine and Biology, (A. R. Karpf , ed.), DOI 10,2013, 754,215-232.
30. Poulain, AJ. and Barkay, T. Microbial Methylmercury Synthesis from Inorganic Mercury in Nature, Science, 2013, 339, 1280-1281.
31. Rutten, B and Mill J. Epigenetic Mediation of Environmental Influences in Major Psychotic Disorders. Schizophrenia Bulletin,2009, 35(6), 1045-1056.
32. Saito et al. Epigenetic Therapy Upregulates the Tumor Suppressor MicroRNA-126 and its Host Gene EGFL7 in Human Cancer Cells. Biochemical and Biophysical Research Communications. 2009, 379, 726–731.
33. Shukla S, Velazquez J, French S, Lu S, Ticku M, and Zakhari S. Emerging Role of Epigenetics in the Actions of Alcohol, Alcoholism. Clinical and Experimental Research**.** 2008, 32 (9), 1525-1534.
34. Song C. et al. Environmental Neurotoxic Pesticide Increases Histone Acetylation to Promote Apoptosis in Dopaminergic Neuronal Cells:Relevance to Epigenetic Mechanisms of Neurodegenerations. Mol. Pharmacol. 2010, 77, 621-632.
35. Stein RA. Understanding of Epigenetics Deepens. Genetic Engineering & Biotechnology News, January 15, 2014, 5 pages.

36. Sun et al. Modulation of Histone Methylation and MLH1 Gene Slencing by Hexavalent C Chromium. Toxicol Appl Pharmacol,2009, 237(3), 258–266.
37. Suva, ML, Riggi, N. and Bernstein, BE. Epigenetic Reprogramming in Cancer. Science. 2013, 339, 1567- 1570.
38. Taguchi et al. Effects of Cadmium on DNA- (Cytosine-5) Methyltransferase Activity and DNA Methylation Status During Cadmium-induced Cellular Tansformation, Experimental Cell Research. 2003, 286, 355–365.
39. Takahaski Y, Kondo K, Hirose T, Nakagawa H, Tsuyuguchi M, Hashimoto M, Sano T, Ochiai A, Monden Y. Microsatellite Instability and Protein Expression of the DNA Mismatch Repair Gene, hMLH1, of Lung Cancer in Chromate-Exposed Workers, Mol Carcinogen, 2005, 42, 150-158.
40. Uden, PC. Chlorinated Acids and Chloral in Drinking Water. J Am Water Works Assoc. 1983, 75,524-527.
41. Waddington, CH.The Epigenotype.Endeavour, 1942, 1, 118-20.
42. Zeisel S. Epigenetics Mechanisms for Nutrition Determinants of Later Health Outcomes. Am J Clin Nutr, 2009, 89, 1488S-1493S.
43. Zhao CQ, et al. Association of Arsenic-induced malignant Transformation with DNA Hypomethylation and Aberrant Gene Expression. Proc Natl Acad Sci USA, 1997, 94, 10907-10912.

Chapter 5

Impact of Heavy Metal Pollution on Freshwater *Coelatura* species (*Mollusca: Bivalvia: Unionidae*) and the Ecosystem of the River Nile In Egypt

***Faiza M El Assal, Salwa F Sabet, kohar G Varjabedian and Mona F Fol**
Department of Zoology, Faculty of Science, Cairo University, Giza, Egypt, 12613
***Corresponding author:** faizaelassal@yahoo.com

Abstract

The Knowledge of heavy metal concentrations in aquatic species is important with respect to genetic variation and extinction of some species and loss of biodiversity in the ecosystem of rivers and lakes. In the present study, we used random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) to examine the genetic diversity of five *Coelatura* species (*C. aegyptiaca*, *C. prasidens*, *C. canopicus*, C. *gaillardoti* and *C. parreyssi*) that were collected for one year, from the River Nile at two polluted locations (El-Kanater, Qalyoubia governorate and Tura, Cairo governorate, Egypt).

We used different short primers in RAPD-PCR and we then investigated the genetic analysis of the results.

RAPD-PCR and the similarity index (D) showed that the five studied species could be classified into only three different species, namely *C. aegyptiaca, C. canopicus* and *C. parreyssi.*

Therefore, the concentration of six heavy metals (copper, cobalt, nickel, manganese, lead and iron) was determined in the soft parts of these bivalves to assess the impact of heavy metal pollution on the freshwater ecosystem. Determined metal concentrations in the *Coelatura* species tissues were found to be higher than the permissible limits, indicating that heavy metals might play an important role in the genetic of *Coelatura* species as well as they may be the cause of the extinction of some species and the loss of biodiversity in the ecosystem of the River Nile.

Keywords

Coelatura, C. aegyptiaca, C. prasidens, C. canopicus, *C. gaillardoti, C. parreyssi*, taxonomy, RAPD-PCR, genetic variation, heavy metals, pollution,

1. Introduction

Pollution of the freshwater environments by heavy metals due to increased action of flowing out discharge from various industries has received considerable attention in that it is able to influence freshwater organisms, leading to modify their genetic diversity (Hochwald and Bauer, 1988; Ashraj, 2005; Al-Weher, 2008; Bala *et al,* 2008; Obasohan *et al,* 2008; El-Assal

and Fol, 2011 and Kamaruzzaman *et al,* 2011). Pollution affects adversely organisms and could be the cause of the genetic variation of some species.

Metal exposure was found to lead to various types of DNA damages and alteration of genetic patterns within populations (Bishop and Cook, 1981 and Klerks and Weis, 1987) and also, DNA damage may indicate levels of metal toxicity.

In Egypt, *Coelatura* species showed great argument on their taxonomy, and their number ranged from 1 to 14 species in various studies (Ibrahim *et al*,1999; Sleem and Ali, 2008 and Graf and Cummings, 2007) which consequently lead to questionable taxonomy. Therefore, in the present study, we used RAPD-PCR method to resolve the conflict on the taxonomical status of some *Coelatura* species from the River Nile in Egypt and to discuss the effect of metal pollution in this respect.

On the other hand, *Unionidae* are declining at a catastrophic rate worldwide. They are threatened by a number of factors among which industrial and human activities inducing environmental pollution (Williams *et al,* 1993), pointing toward impending mass extinction. The significant loss of biodiversity may permanently alter ecosystem functioning in rivers and lakes as well as alter the rate of ecological processes (Hastie *et al*, 2003).

Metal pollution of freshwater sources appears to be the main cause of the endangerment of freshwater mussels which are endangered nowadays worldwide and it is possible that high amounts of metals are toxic and could be a contributing threatening factor (Wang *et al,* 2010). Therefore, it is important to estimate the accurate levels of trace elements in some mussels' species (*Coelatura* species as example) to assess the impact of heavy metals on their genetic variation and on the loss of biodiversity in the ecosystem of the River Nile in Egypt.

2. Material and Methods

2.1 Collections of samples

The *Coelatura* species (*C. aegyptiaca, C. prasidens, C. gaillardoti*, *C. canopicus* and *C. parreyssi*) were collected from the River Nile at two localities, known to be heavy metal polluted (El-Kanater, Qalyoubia Governorate and Tura, Cairo Governorate). Samples were monthly and randomly collected, for one year, from September 2009 to August 2010, then transported to the laboratory, sorted and maintained under the same conditions of food and temperature

2.2 Genetic Study

2.2.1 DNA extraction and RAPD-PCR analysis

Samples of the *Coelatura* species were dissected and their soft parts were preserved in 100% ethyl alcohol at -20 C° until used. Total genomic DNA was extracted from frozen ethanol-preserved mantle using Qiagen Dneasy tissue kit (Valencia, CA, USA) according to the manufacturer's manual. Seven primers were used in the present work, which were previously used in the bivalve RAPD-PCR (Ibrahim *et al,* 2008; Sleem and Ali, 2008 and Yousif *et al,* 2009).

476: 5‘- TTG AGG CCC T – 3‘

477: 5‘ -TGT TGT GCC C – 3‘

478: 5‘ - CGA GCT GGT C – 3‘

479: 5‘ - CTC ATA CGC G – 3‘

483: 5‘ - GCA CTA AGA C– 3‘

486: 5‘ - CCA GCA TCA G – 3‘

487: 5‘ - GTG GCT AGG T – 3‘

Only five primers worked out (UBC 476, UBC 477, UBC 478, UBC 479 and UBC 487). Amplifications were performed by modifying the protocol reported by Williams *et al* (1990). The 25 ìl mixture contained 25 ng of template DNA, 1.5 unit of Taq Polymerase, 10 mMdNTPs, 10 pM primer, and 2.5 ìl of 10x PCR buffer. (Dream Taq Green PCR MasterMix (2X) (Fermentas). Each amplification reaction was performed using a single primer and repeated twice to verify band autosimilarity (Perez *et al,* 1998).

Amplifications were performed in T-personal thermal cycler (Techne, TC-3000G), programmed for 45 cycles of 94° C for 1 minutes., 35° C for 1 minute., and 72° C for 1 minute. An initial denaturation step (3 minutes, 94° C) and a final extension holding (10 minutes, 72° C) were included in the first and last cycles, respectively.

Ten ìl of the reaction products were resolved by 2% agarose gel electrophoresis at 85 volt in 1x TAE (Tris-acetate-EDTA) buffer. The gel was stained with ethidium bromide and photographed by a digital camera under UV transilluminator. For the comparison of the amplified products, population-specific fragments were detected using Gene Ruler 1 kb Plus DNA Ladder from Fermentas.

2.2.2 Molecular data analysis

Molecular data analysis was carried out using gel documentation system (SynGene, GeneTools - File version: 4.02.03, France), for the dendogram and calculation of similarity index of each primer between the studied *Coelatura* species. RAPD amplification products were scored as 0/1 for absence / presence of homologous bands (Abdellatif and Khidr, 2010) and analyses carried out using the NTSYS PC2.0 software (Rohlf,1998).

Similarity coefficient matrix was calculated using Jaccard similarity algorithm (Jaccard, 1908) for RAPD markers. Dendograms were constructed using the UPGMA method, Unweighed pair-Group Method with arithmetical algorithms Averages (Sneath and Sokal, 1973). Genetic diversity was also measured as the percentage of polymorphic bands. The percentage of polymorphic RAPD loci was calculated for each species, as well as the mean and overall value for all species and each primer.

2.3 Heavy metal analysis

2.3.1 Water Analysis

Water and sediment samples collected from the two studied regions were analysed to determine the concentrations of heavy metals, using atomic absorption spectrophotometer model A-Analyst 100 Perkin Elmer. Metals analysed were Copper (Cu), Cobalt (Co), Nickel (Ni), Manganese (Mn), Lead (Pb), and Iron (Fe).

2.3.2 Tissue Analysis (Murphy, 1987)

Mussels were dissected and the soft parts were excised on clean tared pieces of plastic. Wet weights were then determined by the method of Johanson *et al* (1976). Tissues were dried to constant weight, at room temperature, for 24 hours, removed from the plastic pieces and placed in 1.5 ml washed micro centrifuge tubes.

To each tube, 5 ml of piperidine (mole/litre) was added, the tubes were then cooled to room temperature, after which 2 ml of 61% (V/V) HCLO4 was added to the precipitate. After 10 minutes, 7 ml of deionized water was added and mixed. Fifteen minutes later, the tubes were centrifuged for one minute, at 10,000 r.p.m. in a microcentrifuge (Beck Man/ Model J-2, 21). Supernatants were added in aliquots for analysis; using an atomic absorption spectrophotometer, model A-Analyst 100 Perkin Elmer instrumentation laboratories.

Single cuvette attached to an aspiration pump was used to minimize handling of samples and absorption of each ion was integrated for 2 seconds. Metals measured in tissues were Cu, Co, Ni, Mn, Pb and Fe.

2.4 Statistical Analysis

A software computer program SPSS Version 19 was used to test the significance differences between mean values of the different parameters in the studied mussels. One - way ANOVA and MANOVA were employed to find the difference in the ecological analysis at a probability level $P > 0.05$ for insignificant results and $P < 0.05$ and $P < 0.0001$ for significant results.

3. Results

3.1 Genetic studies

Individual amplifications of agarose gel extracted DNA from the mantle of the five studied *Coelatura* species (Fig. 1) were performed using the five primers UBC 476, UBC 477, UBC 478, UBC 479 and UBC 487, in order to determine the genetic relationship between them.

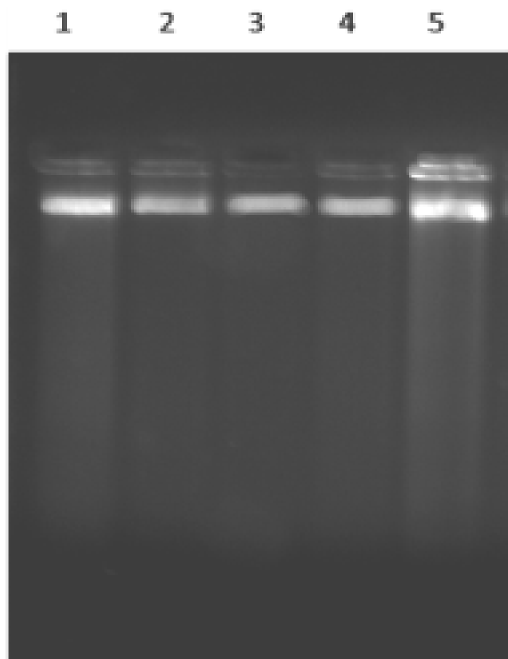


Fig 1: Agrose gel of extracted DNA from the mantle of the five *Coelatura* species under investigation.

1. C. parreyssi,
2. C. aegyptiaca,
3. C. gaillardoti,
4. C. canopicus,
5. C. prasidens

RAPD PCR carried out using the five primers provided strongly amplified fragments (Figs. 2-6).

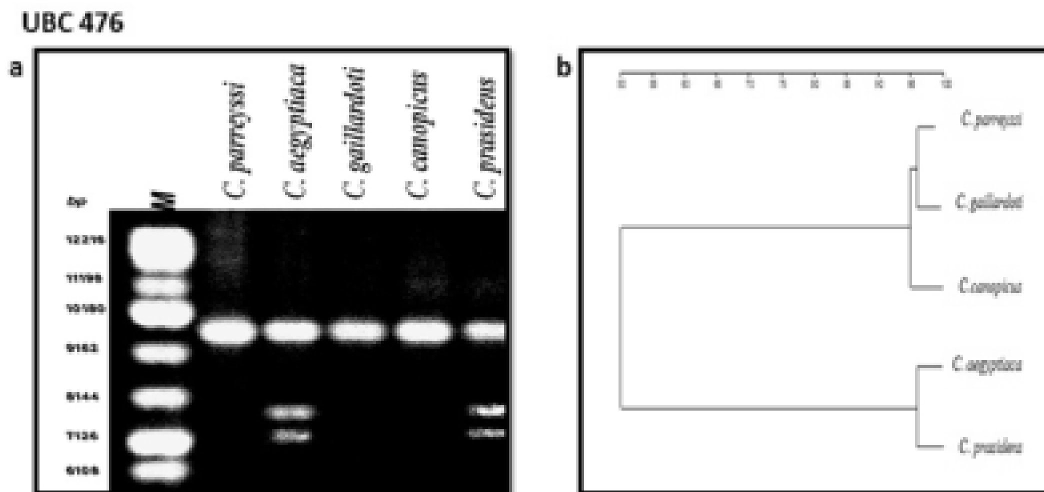


Fig (2): a) RAPD-PCR profiles of the five *Coelatura* species under investigation using primer (UBC 476), and M: 1Kb DNA marker shows one monomorphic band for all studied species and revealed 2 other monomorphic bands for *C. aegyptiaca* and *C. prasidens* b) Dendrogram of primer UBC 476 demonstrating the relationships of the five *Coelatura* species under investigation, based on compiled data set shows that *C. parreyssi, C. gaillardoti* are identical species and *C. canopicus* are closed one also, *C. aegyptiaca* and *C. prasidens* are similar species.

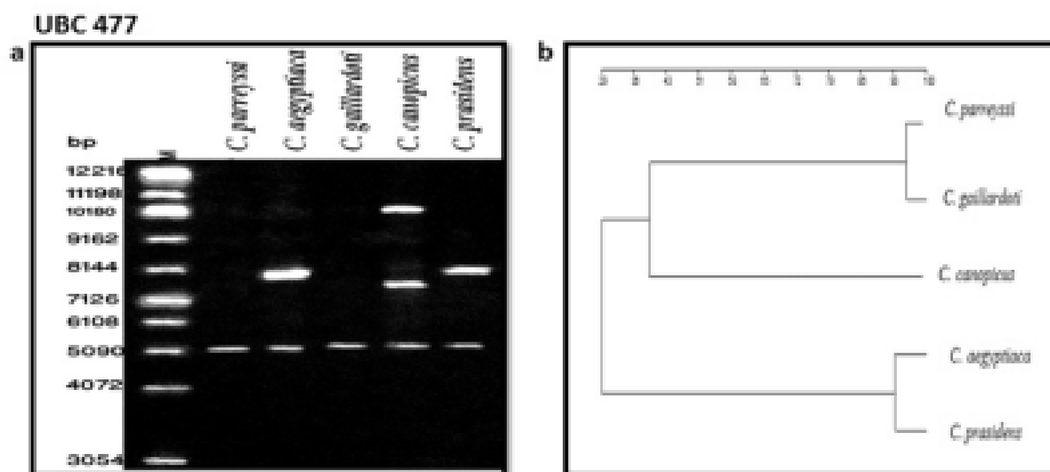


Figure 3. RAPD-PCR analysis of the five *Coelatura* species under investigation using primer (UBC 477) **(a)** gel electrophoresis showing amplification profile of samlpes. M: 1Kb DNA marker. **(b)** Dendrogram demonstrating the similarity relationship between the five *Coelatura* species under investigation

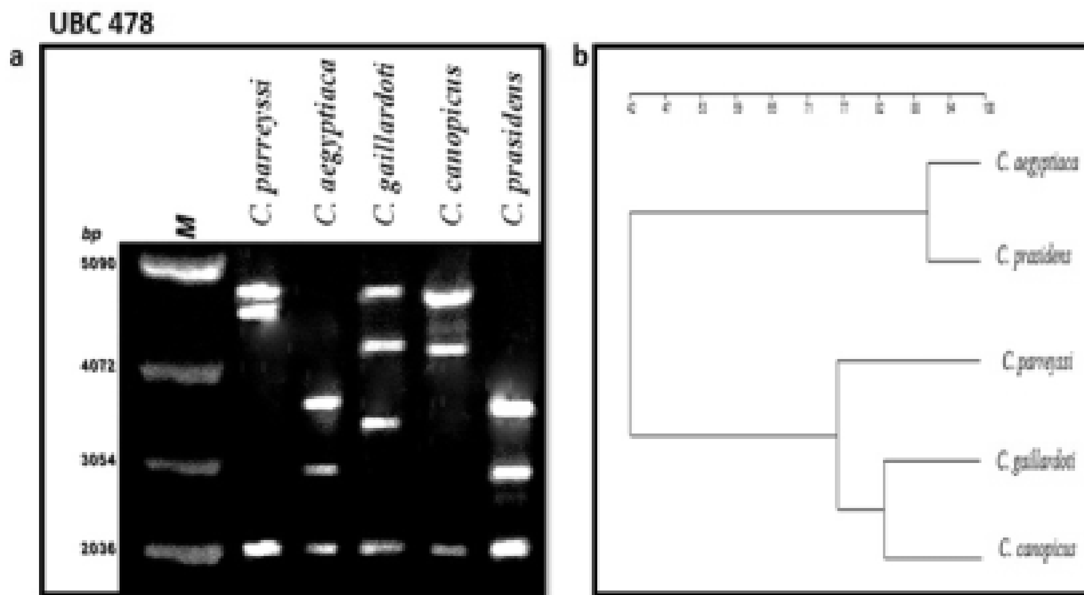


Figure 4. RAPD-PCR analysis of the five *Coelatura* species under investigation using primer (UBC 478) **(a)** gel electrophoresis showing amplification profile of samlpes. M: 1Kb DNA marker. **(b)** Dendrogram demonstrating the similarity relationship between the five *Coelatura* species under investigation

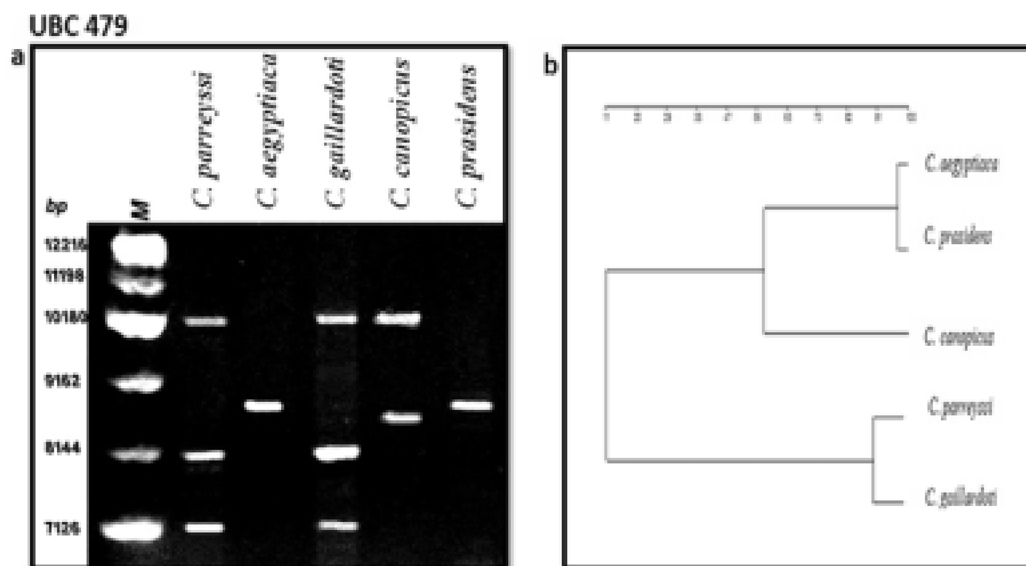


Figure 5. RAPD-PCR analysis of the five *Coelatura* species under investigation using primer (UBC 479) **(a)** gel electrophoresis showing amplification profile of samlpes. M: 1Kb DNA marker. **(b)** Dendrogram demonstrating the similarity relationship between the five *Coelatura* species under investigation

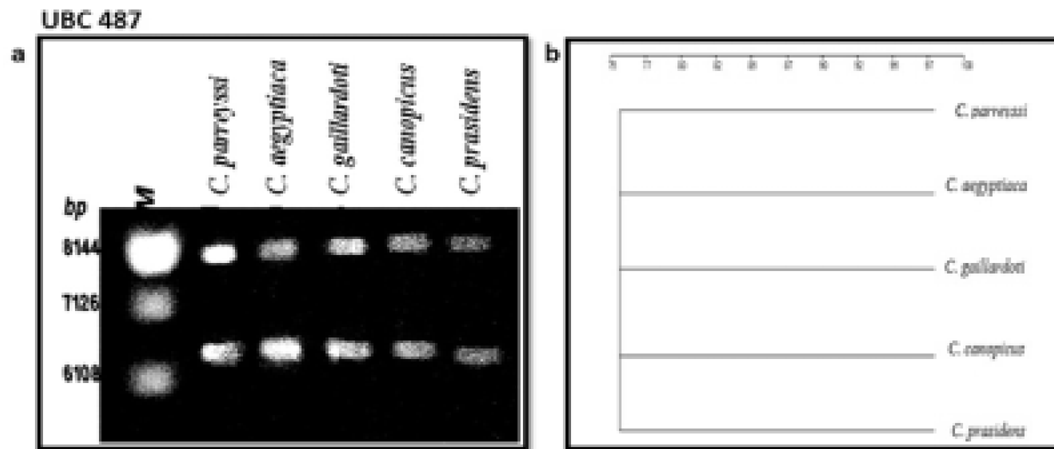


Fig. 6: RAPD-PCR analysis of the five *Coelatura* species under investigation using primer (UBC 487) (a) gel electrophoresis amplification profile of samples M: 1kb DNA marker. (b) Dendrogram demonstrating the similarity relationship between the five *Coelatura* species under investigation.

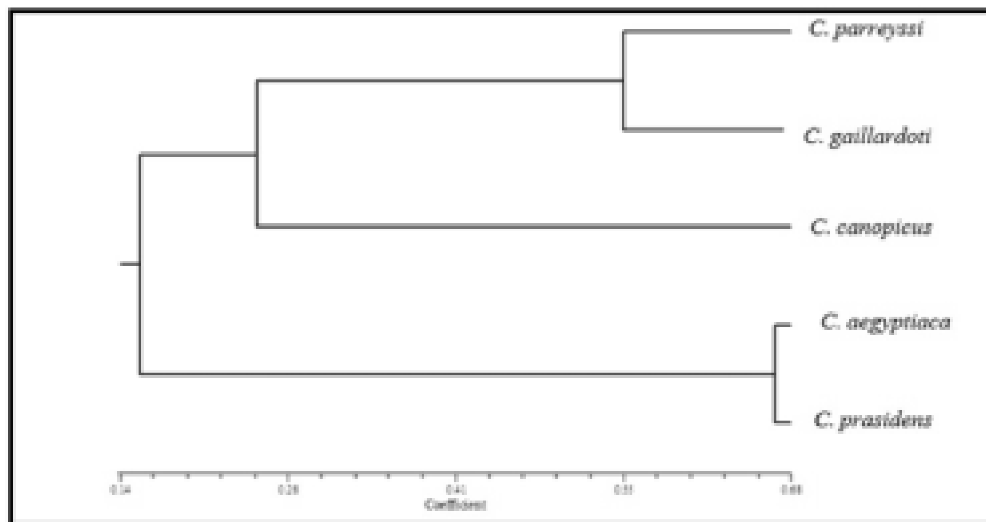


Fig. 7: Dendrogram of cluster analyses of the five *Coelatura* species under investigation according to RAPD data obtained using Jaccard's coefficient and UPGMA method.

Genetic variability was observed among the studied *Coelatura* species. The greatest number of PCR fragments was found with primers UBC 478 and UBC 479 (6-7 bands), while less fragments were obtained with primers UBC 476, UBC 477 and UBC 487 (2-4 bands).The RAPD- PCR analysis was based on the number of bands that were different between any given pair of species (Table 1). Analyses showed natural differences (polymorphism) among *Coelatura* species under investigation.

Primers UBC 477 (Fig.3a) and UBC 479 (Fig.5a) gave monomorphic bands with *C. Parreyssi* and *C. gaillardoti* and as well as with *C. aegyptiaca* and *C. prasidens.* While, *C. canopicus*

revealed some polymorphic bands (Figs. 3a and 5a). Primer UBC 476 gave similar results for *C. aegyptiaca* and *C. prasidens* as with primers UBC 477 and UBC 479, but it showed monomorphic bands for *C. parreyssi, C. gaillardoti* together with *C. canopicus* (Fig. 2a).

However, Primer UBC 478 (Fig. 4a) showed DNA alteration concerning *C. parreyssi, C. gaillardoti* and *C. canopicus*. This DNA alteration might have resulted from mutation or rearrangements at or between oligonucleotide primer binding sites in a genome. Primer UBC 487 (Fig. 6a) revealed monomorphic bands for all five studied *Coelatura* species.

Genetic diversity was also measured as the percentage of polymorphic bands for each primer (Tables 2 and 3). 9.26 % of the bands were polymorphic among the five studied primers. Except primer UBC 487 which revealed no polymorphism, the other primers produced 1 to 6 polymorphic bands. Some RAPD fragments were found to be unique; 1 in *C. parreyssi* and *C. gaillardoti* and 3 in *C. canopicus* (Table 3).

Considering the similarity index (D) of the *Coelatura* species (Tables 5-9), utilizing RAPD-PCR markers, species were considered similar when the (D) value between two species is equal or close to 1, when (D) is distant from 1, the two species were regarded as separate species.

The similarity index (D) between *C. aegyptiaca* and *C. prasidens,* using all studied primers, was close to 1 (0.90-0.97), thus they were the closest species, and were considered one species, *C. aegyptiaca*. While, it was distant from 1 between these two species and the other studied species, except for primer UBC 487 which showed no polymorphism. Also, the (D) value, using the primers UBC 476 and UBC 478, was close to 1 between *C. parreyssi*, *C. gaillardoti* and *C. canopicus*. However, using the primers UBC 477 and UBC 479, (D) was distant from *C. parreyssi* and *C. gaillardoti* were the most closely associated species and may be considered one species, *C. parreyssi*. while *C. canopicus* was somewhat distant and may be regarded as distinct species or subspecies.

The dendrogram analyses, using primers UBC 476, UBC 477 and UBC 479 (Figs 2b, 3b and 5b) confirmed the results obtained with the RAPD profiles and those of the (D) value. *C. aegyptiaca* and *C. prasidens* were the closest species, as well as are *C. parreyssi* and *C. gaillardoti*. while, *C. canopicus* was a separate species. The dendogram using primer UBC 478 showed the same result for *C. aegyptiaca* and *C. prasidens*, while some difference was revealed concerning *C. parreyssi, C. gaillardoti* and *C. canopicus*. The two latter species were the most related species and *C. parreyssi* was somewhat distant (Fig. 3b).According to the Similarity coefficient matrix of all primers (Table 9), the highest (D) value (0.55 and 0.67) was between *C. gaillardoti* and *C. parreyssi* and between *C. prasidens* and *C. aegyptiaca*. while, the lowest D-value (0.12) was recorded between *C. gaillardoti* and *C. aegyptiaca*, *canopicus* and *C. aegyptiaca* and between *C. prasidens* and *C. parreyssi*. This confirms that *C. aegyptiaca* and *C. prasidens* are similar and *C. gaillardoti* and *C. parreyssi* are also similar, while *C. canopicus* is different.

Also, the UPGMA dendogram of all primers (Fig. 7) shows that *C. aegyptiaca* and *C. prasidens* as well as *C. parreyssi* and *C. gaillardoti* are the most closely associated species and *C. canopicus* is the most genetically distinct one. Accordingly, the five *Coelatura* species under investigation may be considered three species only, namely *C. aegyptiaca, C. parreyssi* and *C. canopicus*.

 ISBN: 978-81-928924-3-6

Table 1

Bands Pattern in *C. parreyssi, C. aegyptiaca, C. gaillardoti, C. canopicus* and *C. prasidens* using the five primers

Primers/Species	*C. parreyssi*	*C. aegyptiaca*	*C. gaillardoti*	*C. canopicus*	*C. prasidens*
UBC 476 (Fig 1a)					
1	Band 1 at ~ 9671.16 bp	Band 1 at ~ 9677.96 bp	Band 1 at ~ 9732.50bp	Band 1 at ~ 9739.76 pb	Band 1 at ~ 9759.88bp
2		Band 2 at ~ 7766.91 bp			Band 2 at ~ 7830.31 bp
3		Band 3 at ~ 7261.65 bp			Band 3 at ~ 7302.69 bp
UBC 477 (Fig 2a)					
1	Band 1 at ~ 5157.41bp	Band 1 at ~ 8004.24 bp	Band 1 at ~ 5265.15bp	Band 1 at ~ 10284.48 pb	Band 1 at ~ 8133.93 bp
2		Band 2 at ~ 5167.11 bp		Band 2 at ~ 7655.77bp	Band 2 at ~ 5265.15 bp
3				Band 3 at ~ 5265.15 bp	
UBC 478 (Fig 3a)					
1	Band 1 at ~ 4939.03bp	Band 1 at ~ 3788.28 bp	Band 1 at~ 4952.17 bp	Band 1 at ~ 4890.24bp	Band 1 at ~ 3716.02 bp
2	Band 2 at ~ 4737.70 bp	Band 2 at ~ 3094.70 bp	Band 2 at ~ 4398.09 bp	Band 2 at ~ 4359.32bp	Band 2 at ~ 3061.36 bp
3	Band 3 at ~ 2154.60 bp	Band 3 at ~ 2158.54 bp	Band 3 at ~ 3554.15 bp	Band 3 at ~ 2131.12bp	Band 3 at ~ 2158.54 bp
4			Band 3 at ~ 2158.54 bp		

UBC 479 (Fig 4a)					
1	Band 1 at ~ 10195.41bp	Band 1 at ~ 8834.00 bp	Band 1 at ~ 10272.82 bp	Band 1 at ~ 10319.55bp	Band 1 at ~ 8825.74 bp
2	Band 3 at ~ 8112.54 bp		Band 2 at ~ 8174.51 bp	Band 2 at ~ 8654.18bp	
3	Band 4 at ~ 7153.63 bp		Band 3 at ~ 7188.33 bp		
UBC 487 (Fig 5a)					
1	Band 1 at ~ 8093.80 bp	Band 1 at ~ 8093.80 bp	Band 1 at ~ 8193.97 bp	Band 1 at ~ 8106.52 bp	Band 1 at ~ 8156.88 bp
2	Band 2 at ~ 6542.64 bp	Band 2 at ~ 6542.64 bp	Band 2 at ~ 6556.28 bp	Band 2 at ~ 6583.66 bp	Band 2 at ~ 6529.02 bp bp

Table 2

Total number of bands (monomorphic, polymorphic and percentage of polymorphism) of each primer, in *Coelatura* species under investigation.

Primer	Total number of bands	Monomorphic	Polymorphic	% of polymorphism
UBC 476	3	1	3	66.67 %
UBC 477	4	1	4	75 %
UBC 478	7	1	7	85.7 %
UBC 479	5	0	5	100 %
UBC 487	2	2	2	0 %

* The repeated bands in all species are counted once.

Table 3

Total number of bands for all studied primers (monomorphic, polymorphic, unique) and percentage of polymorphism, revealed by RAPD markers among the five studied *Coelatura* species.

Bands	*C. parreyssi*	*C. aegyptiaca*	*C. gaillardoti*	*C. canopicus*	*C. prasidens*	Total
Amplified	10	11	11	11	11	54
Monomorphic	9	11	10	8	11	49
Polymorphic	1	0	1	3	0	5
Unique	1	0	1	3	0	5
% of polymorphism	10%	0%	9.1%	27.3%	0%	9.26%

Table 4

Similarity index (D) of the Egyptian *Coelatura* specie susing UBC 476 primer.

Species	*C. parreyssi*	*C. aegyptiaca*	*C. gaillardoti*	*C. canopicus*
C. aegyptiaca	0.53			
C. gaillardoti	0.96	0.51		
C. canopicus	0.94	0.52	0.96	
C. prasidens	0.49	0.96	0.49	0.47

Table 5

Similarity index (D) of the Egyptian *Coelatura* species using primer UBC 477.

Species	*C. parreyssi*	*C. aegyptiaca*	*C. gaillardoti*	*C. canopicus*
C. aegyptiaca	0.29			
C. gaillardoti	0.96	0.28		
C. canopicus	0.39	0.30	0.40	
C. prasidens	0.39	0.94	0.38	0.11

Table 6

Similarity index (D) of the Egyptian *Coelatura* species using primer UBC 478.

Species	*C. parreyssi*	*C. aegyptiaca*	*C. gaillardoti*	*C. canopicus*
C. aegyptiaca	0.55			
C. gaillardoti	0.78	0.43		
C. canopicus	0.73	0.35	0.83	
C. prasidens	0.47	0.91	0.39	0.30

Table 7

Similarity index (D) of the Egyptian *Coelatura* species using primer UBC 479.

Species	*C. parreyssi*	*C. aegyptiaca*	*C. gaillardoti*	*C. canopicus*
C. aegyptiaca	0.02			
C. gaillardoti	0.90	0.00		
C. canopicus	0.35	0.58	0.30	
C. prasidens	0.01	0.97	0.008	0.58

Table 8

Similarity index (D) of the Egyptian *Coelatura* species using primer UBC 487.

Species	*C. parreyssi*	*C. aegyptiaca*	*C. gaillardoti*	*C. canopicus*
C. aegyptiaca	0.91			
C. gaillardoti	0.91	0.92		
C. canopicus	0.84	0.85	0.92	
C. prasidens	0.74	0.90	0.77	0.85

^ All studied species show high similarity index (D), ranging from 0.74 to 0.92.

 ISBN: 978-81-928924-3-6

Table 9

Similarity coefficient matrix of all primers calculated by NTSYS of the Egyptian *Coelatura* species.

Species	*C. parreyssi*	*C. aegyptiaca*	*C. gaillardoti*	*C. canopicus*
C. aegyptiaca	0.20			
C. gaillardoti	0.55	0.12		
C. canopicus	0.21	0.12	0.29	
C. prasidens	0.12	0.67	0.19	0.19

3.2 Heavy metal analysis

3.2.1 Nile water and sediment analysis

The mean values of the concentrations of the trace elements measured in the water and sediment of the studied areas (El-Kanater and Tura regions), are given in Table 10.

There was no significant difference (P> 0.05) recorded in Cu and Fe measured in the water or sediment between both sites, while, significant difference (P < 0.0001) in the concentration of Pb in the sediment and water between the two localities, was recorded. Also, Co concentration in the water showed significant difference (P< 0.0001) between both sites. Ni and Mn revealed, too, significant difference (P< 0.0001) in the sediment between the two regions.

Trace elements recorded in the Nile water of both regions were in the permissible levels for Cu, Mn and Fe, while the levels of Co, Pb and Ni exceeded these levels (Table 10).

The concentrations of the different studied heavy metals in water of the two locations were in the following decreasing order:

Tura region: Fe > Co > Ni >Mn>Pb> Cu

El-Kanater region: Fe > Co >Ni >Mn>Pb> Cu

Metal concentrations in the sediment of the two locations were in the following sequence:

Tura region: Fe >Mn> Ni >Pb> Co > Cu

El-Kanater region: Fe >Mn> Co > Ni > Cu >Pb

3.2.2 Tissue analysis

There was a great variation in the amount of the trace elements accumulated in the different soft parts of the studied mussels (Tables 11-14).

In general, significant difference was recorded in the concentration of the studied heavy metals in the different soft parts, between the studied *Coelatura* species (P<0.05, P<0.0001), at the two localities under investigation, except in some instance.

Heavy metals analyzed in all tissues of the three studied *Coelatura* species exceeded the permissible levels according to WHO (1989) and FAO/WHO (1999).

Table10

Concentration of heavy metals (in ppm)in water and sediment of the River Nile at El-Kanater and Tura regions, and the permissible levels in water according to the WHO (2008, 1996).

Metals / Parameters	Pb	Cu	Co	Ni	Mn	Fe
Sediment at Tura	5.24± 0.53	3.34± 0.2	3.9± 0.72	5.32± 0.43	198.7±12.2	303.26±60.7
Sediment at El-Kanater	2.7± 0.4	3.2± 0.19	3.7± 0.47	3.64± 0.48	234.67± 10.6	245.08 ±20.87
P value*	*P< 0.0001	P >0.05	P >0.05	*P< 0.0001	*P< 0.0001	P >0.05
Water at Tura	0.05±0.01	0.022± 0.008	0.22± 0.13	0.06±0.012	0.052±0.01	0.26± 0.03
Water at El-Kanater	0.032± 0.008	0.03±0.01	0.09±0.007	0.05±0.005	0.04±0.004	0.14± 0.076
P value*	*P <0.0001	P >0.05	*P <0.0001	P >0.05	P >0.05	P >0.05
Permissible levels of water	0.01	2	0.001-0.002	0.02	0.4	1

*Significant at P< 0.0001 and insignificant at P <0.05

 ISBN: 978-81-928924-3-6

Table 11

Mean concentrations of the heavy metals in the foot of *C. aegyptiaca, C. parreyssi* and *C. canopicus* in g /kg at Tura and El-Kanater regions ± standard deviation.

Regions	Tura region			P- value	El-Kanater region			P- value	*Permissible
Species / Heavy metals	*C. aegyptiaca*	*C. canopicus*	*C. parreyssi*		*C. aegyptiaca*	*C. canopicus*	*C. parreyssi*		levels in mg/kg
Lead	7.3± 0.80	5.93±0.78	13.1±3.1	*** P<0.0001	3.73 ± 0.24	9.1± 0.5	12± 1.3	*** P<0.0001	0.25 (0.00025 g)
Copper	13±1.7	13.65±2.93	8.34±2.28	*** P<0.0001	9.35±0.83	8± 0.7	6.4±1.23	*** P<0.0001	3 (0.003 g)
Cobalt	6± 1	4.57±2.18	5.36±2.2	P>0.05	0.83± 0.1	3.1± 0.2	5.5± 0.67	*** P<0.0001	-
Nickel **	6.79±1.26	4±2.58	0.54±0.18	*** P<0.0001	3.64±0.52	3.17± 0.54	3.84±0.7	P>0.05	0.5-1.0 (0.0005-0.001 g)
Manga-nese	164.4 ± 17.9	532.39 ±136.56	425.2 ±12.79	*** P<0.0001	223.8± 24.2	263± 37.2	306.1 ±86.3	P>0.05	2-9 (0.002 -0.009 g)
Iron	654±85.4	568.66 ±123.5	472±15.7	*** P< 0.05	278.2±24	339.3 ±34.3	453.3 ±200.2	P>0.05	43 (0.043 g)

Permissible levels of heavy metals according to FAO/WHO, (1999).*

* * Permissible levels of Ni according to WHO (1989). * * * Significant at P< 0.05, P< 0.0001and insignificant at P>0.05

Table 12

Mean concentrations of the heavy metals in the mantle of *C. aegyptiaca, C. parreyssi* and *C.canopicus* in g /kg at Tura and El-Kanater regions ± standard deviation.

Regions	Tura region			P- value	El-Kanater region			P- value	*Permissible
Species / Heavy metals	*C. aegyptiaca*	*C. canopicus*	*C. parreyssi*		*C. aegyptiaca*	*C. canopicus*	*C. parreyssi*		levels in mg/kg
Lead	3.8± 0.74	3.3±0.76	7.63±2.13	*** P<0.0001	2.59± 0.7	4.5± 1	9.4± 1.1	*** P<0.0001	0.25 (0.00025 g)
Copper	10.4±1.24	7.22±1	7.15±0.89	*** P<0.0001	3.9±0.34	3.94±0.3	3.99±0.4	*** P<0.5	3 (0.003 g)
Cobalt	7± 1	11.69 ± 1.52	14.56 ± 2.4	*** P<0.0001	2.57± 0.55	3.8± 0.83	5.1± 0.7	*** P<0.0001	-
Nickel **	11.57± 3.2	11±2.5	7.9±0.69	*** P<0.05	6.13± 1.3	4.3±0.4	2.5±0.6	*** P<0.0001	0.5-1.0 (0.0005-0.001 g)
Manga-nese	392.94 ± 19.8	475.3 ± 70.7	568.2 ±104.7	*** P<0.0001	649±82.9	376.7±43	178± 58.4	*** P<0.0001	2-9 (0.002 -0.009 g)
Iron	238.8±31.7	481.6 ±74.27	473.8 ±47.26	*** P< 0.0001	638.4± 54.6	511.86±40	356.5 ±36.2	*** P<0.0001	43 (0.043 g)

Permissible levels of heavy metals according to FAO/WHO, (1999).*
* * Permissible levels of Ni according to WHO (1989). * * * Significant at P< 0.05, P< 0.0001and insignificant at P>0.05

 ISBN: 978-81-928924-3-6

Table 13

Mean concentrations of the heavy metals in the gills of *C. aegyptiaca, C. parreyssi* and *C.canopicus* in g /kg at Tura and El-Kanater regions ± standard deviation.

Regions	Tura region			P- value	El-Kanater region			P- value	*Permissible
Species / Heavy metals	*C. aegyptiaca*	*C. canopicus*	*C. parreyssi*		*C. aegyptiaca*	*C. canopicus*	*C. parreyssi*		levels in mg/kg
Lead	2.35± 0.78	2.94±0.62	2.54±0.58	P<0.05	4.85± 0.56	5.33± 1.1	7.27± 1.1	*** P<0.0001	0.25 (0.00025 g)
Copper	11.9±0.98	13.45 ±1.96	10.98±1	*** P<0.05	5.6±1.2	5.32±0.7	5.9±0.44	*** P<0.5	3 (0.003 g)
Cobalt	9.99± 1.5	5.45± 0.79	3.77±1.2	*** P<0.0001	8.5± 1	7.3± 0.76	9.3± 1.1	*** P<0.0001	-
Nickel **	11.2± 0.86	9.7±1.2	11±0.47	*** P<0.05	3.6± 0.7	5.94±1.2	6.67±0.44	*** P<0.0001	0.5-1.0 (0.0005-0.001 g)
Manga-nese	282.02 ± 17.63	498.1 ±72.8	142.78 ±25.75	*** P<0.0001	136.56 ±5.25	120.75±41	447 ±26.54	*** P<0.0001	2-9 (0.002 -0.009 g)
Iron	152.47 ±23.375	105.83 ±81.9	144.49 ±24.78	P<0.05	155.98 ±12.32	224.81 ±30.66	261.8 ±25.49	*** P<0.0001	43 (0.043 g)

Permissible levels of heavy metals according to FAO/WHO, (1999).*
* * Permissible levels of Ni according to WHO (1989). * * * Significant at P< 0.05, P< 0.0001and insignificant at P>0.05

Table 14

Mean concentrations of the heavy metals in the digestive tissues of *C. aegyptiaca, C. parreyssi* and *C.canopicus* in g /kg at Tura andEl-Kanater regions ± standard deviation.

Regions	Tura region			P- value	El-Kanater region			P- value	*Permissible
Species / Heavy metals	*C. aegyptiaca*	*C. canopicus*	*C. parreyssi*		*C. aegyptiaca*	*C. canopicus*	*C. parreyssi*		levels in mg/kg
Lead	6.6± 1.3	7.44±1.1	8.45±0.98	P<0.05	5.1± 0.54	3.44± 0.2	3.3± 0.6	*** P<0.0001	0.25 (0.00025 g)
Copper	7.16±1	6.4±0.89	4.9±0.47	*** P<0.0001	4.75±0.24	6.56±0.44	8.4±0.7	*** P<0.0001	3 (0.003 g)
Cobalt	2.55± 1.17	2.3± 0.54	1.1±0.4	*** P<0.05	1.1± 0.07	1.26± 0.3	1.63± 0.3	*** P<0.05	-
Nickel **	7.4± 1	6.1±0.5	2.7±1.3	*** P<0.0001	3.93± 0.6	5.8±0.99	7.3±1.5	*** P<0.0001	0.5-1.0 (0.0005-0.001 g)
Manga-nese	333.3± 33	358 ± 120.8	628.9 ±53.7	*** P<0.0001	153.5±33	115.4 ±15.35	70 ± 14.82	*** P<0.0001	2-9 (0.002 -0.009 g)
Iron	152.88 ±13.17	911.69±85	235.2 ±93.9	P<0.0001	453.2 ± 17.4	669.45 ±27.35	758.7 ±22.4	*** P<0.0001	43 (0.043 g)

Permissible levels of heavy metals according to FAO/WHO, (1999).*
* * Permissible levels of Ni according to WHO (1989). * * * Significant at P< 0.05, P< 0.0001and insignificant at P>0.05

 ISBN: 978-81-928924-3-6

Table 15

Relation between heavy metals and the tissues of the three *Coelatura* species from the two regions of the study.

Fixed parameters	Heavy metals	P-value
Regions (Tura *vs* El-Kanater)	Lead	P< 0.0001
	Copper	
	Cobalt	
	Nickel	
	Manganese	
	Iron	
Species (*C. aegyptiaca vs C. canopicus vs C. parreyssi*)	Lead	P< 0.0001
	Copper	
	Cobalt	
	Nickel	
	Manganese	
	Iron	
Tissues (Foot *vs* Mantle *vs* Gill *vs* Digestive tissues)	Lead	P< 0.0001
	Copper	
	Cobalt	
	Nickel	
	Manganese	
	Iron	
Regions *vs* Species	Lead	P< 0.0001
	Copper	
	Cobalt	
	Nickel	
	Manganese	
	Iron	
	Lead	
	Copper	

Regions *vs* Tissues	Cobalt	P< 0.0001
	Nickel	
	Manganese	
	Iron	
Species *vs* Tissues	Lead	P< 0.0001
	Copper	
	Cobalt	
	Nickel	
	Manganese	
	Iron	
Regions *vs* Species *vs* Tissues	Lead	P< 0.0001
	Copper	
	Cobalt	
	Nickel	
	Manganese	
	Iron	

4. Discussion

Human exploitation of world mineral resources and advances in industrialization has resulted in high levels of heavy metals in the environment (Haggag *et al,* 1999; Salah El-Deen) of the *Coelatura* species,*et al,* 1999 and Zaghloul *et al*, 2000). The aquatic bodies near the industrial and urban areas are more able to accumulate such metals, causing hazardous impact on the freshwater fauna. The impact of metals on different bivalve populations revealed that those inhabiting environments contaminated by heavy metals exhibited a higher allelic diversity (Moraga *et al*, 2002). DNA damage and genetic diversity in aquatic animal populations induced by chemical contaminants have been successfully detected using RAPD method (Nadig *et al*, 1998; Krane *et al*, 1999 and Atienzar *et al*, 2000 and 2002).

RAPD-PCR analysis proved to be helpful in estimating genetic variations among species (Sleem and Ali, 2008). Analyses of the RAPD-PCR showed natural differences or polymorphism among the *Coelatura* species under investigation, and distinguished them to only three species namely, C. *aegyptiaca*, C. *parreyssi* and C. *canopicus* which were also confirmed by dendograms and (D) values. In fact, thorough revision of genus *Coelatura* was needed by applying molecular techniques to reveal the current concept that it represents a lumped species complex, as claimed by Ortmann (1920), Graf (1998) and Graf and Cummings (2007).

The present study shows that C. *aegyptiaca* and C. *prasidens* are closely related and could be considered as one species, C. *aegyptiaca*, which is the type species of the genus *Coelatura*.

 ISBN: 978-81-928924-3-6

Also, C. *parreyssi* and C. *gaillardoti* are closely related and are considered as the same species, *C. parreyssi*, which has advantage over C. *gaillardoti* because of nomenclature priority (Philippi, 1848). On the other hand, C. *canopicus* is somewhat distant from the other studied species and may be considered a separate species or a subspecies. Finally, the similarity coefficient matrix and the UPGMA dendogram of all primers confirmed that the five *Coelatura* species under investigation should be classified into three species only namely, C. *aegyptiaca*, C. *parreyssi* and C. *canopicus*. Thus, assessing the genetic diversity of populations could be a valuable addition to more traditional tools for determining the effects of environmental pollution on aquatic ecosystems (Nevo *et al,*1986; Bickham and Smolen, 1994; Nadig *et al,* 1998).

Primer UBC 478 showed DNA alteration concerning C. *parreyssi*, C. *gaillardoti* and *C.canopicus*. The gain/loss of RAPD bands may be related to DNA damage, mutation or structural rearrangements induced by genotoxic agents affecting the primer sites (Atienzar *et al,* 2002). Mutation may be due to quantitative or qualitative changes or rearrangement of the genetic material, most probably due to metal (Pb, Mn, Cu, Fe, Co and Ni) pollution of the environment, recorded in the two localities of the study. The concept that genetic patterns within populations may be altered by exposure to contaminants was reported by Bishop and Cook (1981), Klerks and Weis (1987), and Abdul-Aziz (2012). The evidence for pollutant to induce genotoxicity has been also determined by several authors (Belfiore and Anderson, 2001; Coughlan *et al,* 2002; Moraga *et al,* 2002; De Wolf *et al,* 2004 and Liyan *et al,* 2005).

Formi (1994) and Reid *et al* (1994) reported Cu, Fe, Cd and Ni as mutagenic agents. These metals have the tendency to bind to phosphates and wide variety of organic molecules including base residues of DNA, which can lead to mutations by altering structures of DNA (Wong, 1988) or modifying the genetic diversity of populations. Also, exposure of mussels in the field to water polluted by different mixtures of genotoxic contaminants was reported by Izquierdo *et al* (2003) to induce DNA alterations, leading to genetic variation among species and populations. Thus, the conflict in the taxonomy of *Coelatura* species in the different studies is most probably due to the environmental pollution with heavy metals among other factors. Heavy metals analysed in all tissues of the studied *Coelatura* species exceeded the permissible levels according to WHO (1989) and FAO/WHO (1999).

The numbers of threatened aquatic species and species extinctions increase at an alarming rate (Baillie *et al,* 2004). Molluscs are one of the most threatened major taxonomic groups worldwide (Lydeard *et al,* 2004). Within this group, the unionids are highly threatened throughout their distribution (Bogan, 1993) and are declining globally due to alteration in habitat, decline and extinction of fish host populations, pollution and environmental changes, pointing toward impending extinction. They are the most imperiled group of species and many species became extinct in several parts of the world including Egypt, while others are threatened or endangered. The loss of benthic biomass may result in large scale alterations of freshwater ecosystem processes and functions (Ricciardi *et al*, 1998).

Little information is available about the effect of frequent exposure to metals on mussels; it is possible that higher metal amounts than required could be a contributing factor to the

extinction of some mussel species and genetic variation of some other species. In fact, the mussel fauna in Egypt is threatened due to heavy metal pollution of the River Nile water and sediment among other factors.

All species of genus *Unio* which were used to live in the River Nile are today extinct (Ibrahim *et al*, 1999). Only fossils were recorded by these authors from El Fayoum, Komombo, Idfu and Isna *i.e* from Upper Egypt. Although, some investigators (Aboul-Dahab, 2002 and Ramadan, 2003) have referred to living *Unio* specimens in the River Nile in Lower Egypt. But, this is uncertain and needs to be thoroughly revised and the occurrence of *Unio* species in Egypt is still doubtful.

In general, studies on heavy metals are important in two main aspects, the public health point of view and the aquatic environment conservation. Heavy metals are present in the aquatic environment where they can accumulate along the food chain. Moreover, small amounts of absorbed heavy metals are either stored in a metabolically available form for essential biochemical processes or detoxified into metabolically inert forms and held in the body either temporarily or permanently (Hashm i*et al*, 2002). Thus, determination of chemical quality of aquatic organisms, particularly the content of heavy metals is extremely important.

References

1. Abdellatif, K. F. and Khidr, Y. A., Genetic diversity of new maize hybrids based on SSR markers as compared with other molecular and biochemical markers, J. Crop Sci. Biotech., 2010, 13(3): 139-145.
2. Abdul-Aziz, K. K., The Physiological Status and Genetic Variations of the Bivalve, *Pinctada radiata* Affected by Environmental Pollution. Research. Journal of Pharmaceutical, Biological and Chemical Sciences, 2012, 3(3): 277-291.
3. Aboul-Dahab, H. M., Cellular responses of the molluscan host, *Unio abyssinicus* infected by two new haplosporidian parasites with emphasis on the morphological characteristics of the host haemolymph. Egyptian Journal of Zoology, 2002, 38: 149-170.
4. Al-Weher, S. M., Levels of Heavy metals Cd, Cu and Zn in three fish species collected from the Northern Jordan Valley, Jordan. Jordan Journal of Biological Sciences, 2008, 1(1):41-46.
5. Ashraj, W., Accumulaation of heavy metals in kidney and heart tissues of *Epinephelus microdon* fish from the Arabian Gulf. Environ. Monit.Assess., 2005, 101 (1- 3):311-316.
6. Atienzar, F. A.; Cordi, B.; Donkin, M. E.; Evenden, A. J.; Jha, A. N. and Depledge, M. H., Comparison of ultraviolet-induced genotoxicity detected by random ampilified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmate*. Aquat. Toxicol., 2000, 50:1-12.
7. Atienzar, F. A.; Venier, P.; Jha, A. N. and Depledge, M. H., Evaluation of random ampilified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations, Mutat. Res., 2002, 521:151-163.
8. Baillie, J. E. M.; Hilton-Taylor, C. and Stuart, S. N., IUCN red list of threatened species. A global species assessment, 2004, 1-217.
9. Bala, M.; Shehu, R. A. and Lawal, M., Determination of the level of some heavy metals in water collected from two pollution- Prone irrigation areas around Kano Metropolis, Bayero J. P. and Appl. Sci., 2008, 1(1): 36-38.
10. Belfiore, N. M. and Anderson, S. L., Effects of contaminants on genetic patterns in aquatic organisms: a review, Mutat. Res., 2001, 289:97-122.

 ISBN: 978-81-928924-3-6

11. Bickham, J. W. and Smolen, M. J., Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environ. Health Perspect, 1994, 102:25-28.
12. Bishop, J. A. and Cook, L. M., Genetic consequences of man made change, Academic Press, London, 1981, pp. 209.
13. Bogan, A. E., Freswater bivalve extinctions search for a cause *(Mollusca: Unionida),* Amer. Zool., 1993, 33: 599-609.
14. Coughlan, B. M.; Hartl, M. G. J.; O'Reilly, S. J. Sheehan, D.; Morthersill, C.; Van Pelt, F. N. A. M.; O'Halloran, J. and O'Brien, N. M., Detecting genotoxicity using the Comet assay following chronic exposure of Manila clam *Tapes semidecussatus* to polluted estuarine sediments. Mar.Pollu. Bull., 2002, 44(12):1359-1365.
15. De Wolf, H.; Blust, R. and Backeljan, T., Population genetic structure of *littorina littorea* (*Mollusca: Gastropoda*) along population gradient in the scheldt Estuary the Netherlands using RAPD analysis. Sci. Total Environ., 2004, 325(1-3): 59-69.
16. El-Assal, F. and Fol, M., *Corbicula species* (*Bivalvia: Veneroida*), from the River Nile in Egypt as bioindicators of pollution. Environmental Pollution Ecological Impacts Health Issues and Management, 2011, 82-91.
17. FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization), Evaluation of certain food additives and contaminants. Expert Committee on Food Additives. WHO Technical Report Series, 1999, No. 884, p. 7.
18. Formi, A., Comparison of chromosome aberrations and micronuclei in testing genotoxicity in humans. Toxicol. Lett., 1994, 72:185-190.
19. Graf, D. L., Sympatric speciation of freshwater mussels (*Bivalvia: Unionoidea*): a model. American Malacological Bulletin, 1998, 14: 35-40.
20. Graf, D. L. and Cummings, K. S., Preliminary review of the freshwater mussels (*Mollusca: Bivalvia: Unionoida*) of Northern Africa, with an emphasis to the Nile. J. Egypt. Ger. Soc. Zool., 2007, 53D: 89-118.
21. Haggag, A. M.; Marie, M. A. S. and Zaghloul, K. H., Seasonal effects of the industrial effluents on the Nile catfish; *Clarias gariepinus.* J. Egypt. Ger. Soc. Zool., 1999, 28(A): 365-391.
22. Hashmi, M. I.; Mustafa, S. and Tariq, S. A., Heavy metal concentrations in water and tiger prawn (*Penaeus monodon*) from grow-out farms in Sabah, North Borneo. Food Chemistry, 2002, 79: 151-156.
23. Hastie, L. C.; Cosgrove, P. J.; Ellis, N. and Gaywood, M. J., The Threat of Climate Change to Freshwater Pearl Mussel Populations. Ambio, 2003, 32: 40-43.
24. Hochwald, S. and Bauer, G., GutachtenzurBestandssituation und zumschutz der Bachmuschel *Unio crassus* (Phil.) in Nordbayen Fischer und Teichwirt, 1988, 12:366-371.
25. Ibrahim, A. M.; Bishai, H. M. and Khalil, M. T., Freshwater Molluscs of Egypt: Publication of National Biodiversity unit, 1999, No.10. Egyptian Environmental Affairs Agency, Dept. of Nature protection.
26. Ibrahim, A. M.; Aly, R. H.; Kenchington, E. and Ali, T. G., Genetic polymorphism among five populations of *Pinctada radiat*a from the Mediterranean coast in Egypt indicated by RAPD-PCR technique. Egypt. J. Zool., 2008, 50: 467-477.
27. Izquierdo, J. I.; Machado, G.; Ayllon, F.; d'Amico, V.; BalaVallarono, E.; Elias, R. and Garcia-Vazquez, E., Assessing pollution in coastal ecosystems: a preliminary survey using the micronucleus test in the mussel *Mytilus edulis*, Ecotoxicol. Env.Safty., 2003, 55: 24-29.

28. Jaccard, P., Nouvelles recherches sur la distribution florale. Bull. Soc. Vaudoise Sci. Nat., 1908, 44: 223-270.
29. Johanson, C. E.; Reed, D. J. and Woodbury, D. M., Brain Research, 1976, 116: 35-48.
30. Kamaruzzaman, B. Y.; MohdZahir, M. S.; Akbar John, B.; Jalal, K. C. A.; Shahbudin, S.; Al-Barwani, S. M. and Goddard, J. S., Bioaccumulation of some heavy metals by green-lipped mussels *Perna viridis* (Linnaeus, 1758) from Pekan, Pahang, Malaysia. International Journal of Biological Chemistry, 2011, 5(1): 54-60.
31. Klerks, P. L. and Weis, J. S., Genetic adaptation to heavy metals in aquatic organisms: a review. Environ. Pollut., 1987, 45: 173-205.
32. Krane, D. E.; Sternberg, D. C. and Burton, G. A., Randomly amplified polymorphic DNA profile-based measures of genetic diversity in crayfish correlated with environmental impacts. Environmental Toxicology and Chemistry, 1999, 18: 504–508.
33. Liyan, Z.; Ying, H. and Guangxing, L., Using DNA damage to monitor water environment. Chinese J. Oceanol.Limnol., 2005, 23(3): 340-348.
34. Lydeard, C.; Cowie, R. H.; Ponder, W. F.; Bogan, A. E.; Bouchet, P.; Clark, S. A.; Cummings, K. S.; Frest, T. J.; Gargominy, O.; Herbert, D. G.; Hershler, R.; Perez, K. E.; Roth, B.; Seddon, M.; Strong, E. E. and Thompson, F. G., The global decline of nonmarine mollusks. Bioscience, 2004, 54, 321-330.
35. Moraga, D.; Mdelgi-Lasram, E; Romdhane, M. S.; El Abed, A.; Boutet, I.; Tanguy, A. and Auffret, M., Genetic responses to metal contamination in two clams: *Ruditapes decussates* and *Ruditapes philippinarum*. Mar. environ., 2002, Res., 7:521-525.
36. Murphy, V. A., Method of determination of Sodium, Potassium, Calcium, Magnesium, Chloride, and Phosphate in the rat choroids plexus by Flame Atomic Absorption and Visible Spectroscopy Analytical Biochemistry, 1987, 161: 144-151.
37. Nadig, S. G.; Lee, K. L. and Adams, S. M., Evaluating alterations of genetic diversity in sunfish populations exposed to contaminants using RAPD assay. Aquatic Toxicology, 1998, 43, 163–178.
38. Nevo, E.; Noy, R.; Lavie, B.; Beiles, A. and Muchtar, S., Genetic diversity and resistance to marine pollution. Biological Journal of the Linnean Society, 1986, 29, 139–144.
39. Obasohan, E. E.; Oronsaye, J. A. O. and Eguavoen, O. I., A comparative Assessment of the heavy metal loads in the tissues of a common catfish (*Clarias gariepinus*) from Ikpoba and Ogba Rivers in Benin City, Nigeria. African Scientist, 2008, 9(1): 13-23.
40. Ortmann, A. E., Correlation of shape and station in freshwater mussels (naiads) proceedings of the American Philosophical Society, 1920, 59(4): 269-312.
41. Perez, T.; Albornoz, J. and Dominguez, A., An evaluation of RAPD fragments reproducibility and nature. Molecular Ecology, 1998, 7: 1347-1357.
42. Philippi, R. A., Abbildungen und Beschreibungen never Oder wenig gekannter conchylien, II and III Kassel., 1848.
43. Ramadan, S. A., A comparison of the population structure of the water mite *Unionicola tetrafurcatus* inside two molluscan hosts from the River Nile, Egypt. Egyptian Journal of Zoology, 2003, 41: 229-247.
44. Reid, T. M.; Dunn, J. F. and Loeb, L. A., Mutagenesis by metal-induced oxygen radicals. Environ. Health Perspect, 1994, 102: 57-61.
45. Ricciardi, A.; Neves, R. J. and Rasmussen, J. B., Impending extinctions of North American freshwater mussels (*Unionida*) following the zebra mussel (*Dreissena polymorpha*) invasion. Journal of Animal Ecology, 1998, 67: 613-619.

46. Rohlf, F. J., NTSYSpc. Numerical taxonomy and multi variate analysis system, 1998, version 2.02c. Exeter Software, New York.
47. Salah El-Deen, M. A.; Khalid, H. Z.; Gamal, O. and Abo-Hegab, S., Concentrations of heavy metals in water sediment and fish in the River Nile in the industrial area of Helwan. Egypt. J. Zool., 1999, 32: 373-395.
48. Sleem S. H. and Ali, T. G., Application of RAPD-PCR in taxonomy of certain freshwater bivalves of genus *Caelatura*. Global Journal of Molecular Sciences, 2008, 3(1):27-31.
49. Sneath, P. H. A. and Sokal, R. R., Numerical taxonomy, Freeman, San Francisco., 1973.
50. Wang, N. I.; Christopher G. I.; Christopher D.; Hardesty, D. K.; May, T. W.; Augspurger, T. R.; Andy D. G.; Eric van, B. M. and Christopher, Sensitivity of Early Life Stages of Freshwater Mussels to Acute and Chronic Toxicity of Lead, Cadmium, and Zinc in Water. Environmental Toxicology and Chemistry, 2010, 29. 9: 2053-63.
51. Williams J. G. K.; Kubelik A. R.; Livak J. K.; Rafalski J. A. and Tingey S.V., DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research., 1990, 18, 6531-6535.
52. Williams, J. D.; Warren, M. L.; Cummings, K. S.; Harris, J. L. and Neves, R. L., Conservation status of freshwater mussels of the United States and Canada. Fisheries, 1993, 18 (9).
53. Wong, P.K., Mutagenicity of heavy metals. Bullet. Environ. Contami. Toxicol., 1988, 40:597-603.
54. World Health Organization (WHO), Heavy metals-environmental aspects. Environment Health Criteria., 1989, No. 85. Geneva, Switzerland.
55. World Health Organization (WHO), Guidelines for drinking-water quality, 1996, 2nd ed. Vol.2. Health Criteria and Other Supporting Information, Geneva.
56. World Health Organization (WHO), Guidelines for drinking-water quality. Geneva, 2008, 3rd ed. Vol.1.
57. Yousif, F.; Ibrahim, A.; Sleem, S.; El Bardicy, S. and Ayoub, M., Morphological and genetic analyses of *Melanoides tuberculata* populations in Egypt. Global Journal of Molecular Sciences, 2009, 4(2), 112-117.
58. Zaghloul, K.H.; Omar, W. A.; Mikhail, W. Z. and Abo-Hegab, S., Ecological and biochemical studies on the Nile fish *Orechromis niloticus* (L.), cultured in different aquatic habitats. Egypt. J. Zool., 2000, 34, 379-409.

Chapter 6

A New Possibility for Separate Identification of Organophosphates and Carbamates

***Tonkopii V.**
Institute of Limnology, Russian Academy of Sciences, Sevastyanov str., 9, St.Petersburg, Russia
***Corresponding author:** tonkopi@hotmail.com

Abstract

It is well known that the cholinesterase of fish's brain is the typical acetylcholinesterase (AChE) with the same substrate specificity. On the other hand, the ChE of some fish's blood plasma has its own specificity. Some years ago for the first time we discovered that only the blood serum of freshwater fish from family of Cyprinidae (blue bream - Abramis ballerus, roach - Rutilus rutilus) contains nonordinary ChE with unusually high sensitivity to organophosphates - dipterex and DDVP and low sensitivity to carbamate neostigmine. This observation is of scientific and practical interest and so the fish's blood plasma ChE of these species was purified for study of kinetic behaviour and sensitivity to antiChE compounds. The sensitivity of enzyme to 45 organophosphates (including sarin, soman and Vx) and carbamates has been determined. The Russian commercial purified lyophilized cholinesterases have been used for comparison: AChE from the erythrocytes of human, butyrylcholinesterase (BuChE) from the horse blood serum and propionylcholinesterases (PrChE) from the hen blood serum and from squid optic ganglion. We investigated the activity of new fish's purified enzyme relatively to a choline and thiocholine esters hydrolysis as a function of substrate concentration. The results of this and the following experiments indicate that the new ChE from the blue bream and roach blood serum can be classified as BuChE, so the velocity of butyrylcholine and butyrylthiocholine hydrolysis is more than other substrates. . The sensitivity of fish's ChE to organophosphates is in 100-2000 times higher than the sensitivity of all types of commercial ChE. On the other hand a new enzyme has an extremely low sensitivity to carbamates. It is very important that with the help of a new purified fish's ChE the separate identification of organophosphorus CW, pesticides and carbamates may be carried out. The extremely low sensitivity of new enzymes to carbamates and very high sensitivity to organophosphates is particularly valuable for these purposes.

Keywords:

Organophosphates, carbamates, detection, cholinesterases.

1. Introduction:

Among many xenobiotics entering aqueous media, anticholinesterase (antiChE) compounds notable for their high toxicity and selectivity are of particular hazard. Many compounds of these

classes are used as pesticides, drugs, chemical warfare agents and many have thus become environmental contaminants. Currently, dozens of pesticides (organophosphorus compounds and carbamates) capable of polluting the aqueous media through the runoff from agricultural lands or as a result of chemical industry accidents are produced. The forthcoming destruction of chemical weapons, a major part of which constitutes antiChE compounds (sarin and soman) can also lead to water pollution. Although the stability of these substances in water is not high (e.g., when compared to organochlorine compounds), certain xenobiotics of these classes are highly toxic for humans, animals, and particularly for hydrobionts. At present, various types of purified commercial cholinesterases (ChE) are widely used to detect antiChE compounds. The detection is based on the property of xenobiotics to lower the activity of enzymes. However, this method is not universal because ChE has low sensitivity to many organophosphorus (OP) and carbamate pesticides [1].

It is known that the cholinesterase of fish's brain is the typical acetylcholinesterase (AChE) with the same substrate specificity and sensitivity to organophosphorus compounds as AChE from human erythrocytes [2]. The fish's blood serum cholinesterase also has been identified as AChE [3]. On the other hand the blood serum of only two freshwater fishes (blue bream - *Abramis ballerus* and roach - *Rutilus rutilus*) contains mainly butyrylcholinesterase (BuChE) with unusually high sensitivity to organophosphorus pesticides - dipterex and DDVP [4]. This is of both scientific and practical interest.

2. Material and Methods:

In this work we studied the substrate specificity, kinetic behaviour and sensitivity of blue bream blood serum ChE for some OP and carbamate pesticides. The earlier unstudied blood serum of blue bream was used as a source of ChE. The fish were collected during autumn - winter period from the Volga pool of the Rybinsk Reservoir. The blood was taken away from fish tail vein and the fibrin clot was separated from the serum. After isolation and purification of blood serum by help of well - known methods, a stabilized lyophilized enzyme with activity of 5 - 10 units per mg of protein was obtained. The following Russian commercial purified lyophilized cholinesterases have been used for comparison: AChE (acetylcholine acetylhydrolase, EC 3.1.1.7) from the erythrocytes of human, BuChE (acylcholine acylhydrolase, EC 3.1.1.8) from the horse blood serum and propionylcholinesterases - PrChE (acylcholine acylhydrolase, EC 3.1.1.8) from the hen blood serum (PrChE-1) and from the squid (*Todarodes pacificus*) optic ganglion (PrChE-2). The kinetics of cholinesterase hydrolysis of different substrates was investigated at pH-7.5 and at 25°C. Acetylcholine iodide (ACh), propionylcholine iodide (PrCh), butyrylcholine iodide (BuCh), acetylthiocholine iodide (AThCh), propionylthiocholine iodide (PrThCh), butyrylthiocholine iodide (BuThCh) were used as substrates. The initial rate of enzyme hydrolysis was measured by the following methods. In the experiments with choline esters it was measured by the potentiometric titration [5] in 1mM of phosphate buffer and 100 mM of potassium chloride. In the experiments with thiocholine esters it was measured by the photometric method of Ellman [6] in the 20 mM of phosphate buffer containing 100 mM of potassium chloride and 0.2 mM5',5'-dithio-bis-(2-nitrobenzoic acid). The maximal rates of enzyme reaction and Michaelis constants (K_m) were obtained by the Lineweaver - Burk method [7] using a computerized production control system.

The irreversible inactivation of the enzyme by OP and carbamates was measured with the help of bimolecular rate constant (K_2) of interaction of the enzymes with inhibitors at pH 7.5 and 25°C in the 20 mM of phosphate buffer, containing 100 mM of potassium chloride and 0.2 M5',5'- dithio-bis - (2-nitrobenzoic acid). In experiments were used the following organophosphates and carbamates. 1. Organophosphates: dipterex, DFP (diisopropylfluorophosphate), dichlorvos (DDVP), paraoxon, phosphamidon, dimethoate, mevinphos, sarin, soman. 2. Carbamates: physostigmine, aminostigmine (2-dimethylaminomethylene-3-dimethylcarbamoyloxypyridine), bizerine (N,N'-hexamethylene-bis-[N-methylcarbamic acid 3 - (2 - dimethylaminomethyl) pyridile ester] tetrahydrochloride), sevine, bis-quaternary carbamate - X-129 (1-[N- (3-[dimethylcarbamoyloxy] - a - picolile) -N,N- dimethylammonio] - 10 - [N- oxyethyl - N,N - dimethylammonio]decabromine).

3. Results:

The curve of the dependence between the hydrolysis rates of substrates by fish ChE (FChE) and the value of pH looks like a bell and has a maximum at pH 8.5. The curves for pH-dependence in the experiments with FChE differed only slightly from these for purified cholinesterases - AChE and BuChE. We investigated the activity of fish ChE (FChE) relatively to a choline and thiocholine esters hydrolysis as a function of substrate concentration. The optimal substrates for FChE are BuCh and BuThCh; the activity of enzyme rises with an increase of substrates concentration. The results of this and the following experiments indicate that the purified ChE of blue bream blood serum can be classified as a BuChE, so the velocity of butyrylcholine and butyrylthiocholine hydrolysis is more than other substrates. At the same time, this type of FChE differs from typical BuChE, so the hydrolysis rate of butyrylcholine by FChE is in 10 - 13 times more rapid as compared to hydrolysis of acetylcholine (Table 1).

Table 1: The kinetic parameters (K_m $_m$M, and relative rate of substrates hydrolysis –V) of different cholinesterases (Rates of hydrolysis expressed as percentages of the acetylcholine rate).

Enzymes	Substrates					
	ACh	PrCh	BuCh	AThCh	PrThCh	BuThCh
V FChE	100	450	1140	500	800	1250
Km	1300	560	170	430	57	16
V AChE	100	70	5	89	19	5
Km	190	260	-	88	160	-
V BuChE	100	173	260	141	127	210
Km	910	550	770	540	510	430

On the other hand the hydrolysis rate of butyrylcholine by BuChE is only in 1.2 - 2.6 more rapid as compared to hydrolysis of acetylcholine. The comparison of Michaelis constants for different

substrates confirms the differences between the FChE, horse blood plasma BuChE and human erythrocytes AChE. The value of K_m for FChE is 5 times less in the case of butyrylcholine and 30times less for butyrylthiocholine as compared with ordinary BuChE. The differences between FChE and other types of ChE are especially appreciable during the study of different inhibitors of cholinesterase. The FChE has a very high sensitivity to some organophosphorus compounds (Table 2).

Table 2: The bimolecular rate constant (k_2 M^{-1} min^{-1}) of interaction of the enzymes with organophosphates and carbamates.

Compounds	Types of cholinesterases				
	AChE	BuChE	PrChE-1	PrChE-2	FChE
Dipterex	$2{\cdot}10^{3}$	$2.6\ 10^{3}$	$1.2\ 10^{3}$	$2\ 10^{4}$	$1.8\ 10^{5}$
DDVP	$1.1{\cdot}10^{4}$	$2.1{\cdot}10^{4}$	$2.3{\cdot}10^{4}$	$2.5{\cdot}10^{5}$	$3.1{\cdot}10^{7}$
Paraoxon	$3{\cdot}10^{6}$	$1.3{\cdot}10^{6}$	$2{\cdot}10^{6}$	$7{\cdot}10^{6}$	$3.4{\cdot}10^{7}$
DFP	$1.1{\cdot}10^{4}$	$4.2{\cdot}10^{6}$	$4.5{\cdot}10^{5}$	$5{\cdot}10^{6}$	$8{\cdot}10^{7}$
Phosphamidon	$5{\cdot}10^{3}$	$5{\cdot}10^{3}$	$5{\cdot}10^{4}$	$8{\cdot}10^{4}$	$8{\cdot}10^{5}$
Dimethoate	$6{\cdot}10^{3}$	$1{\cdot}10^{4}$	$2{\cdot}10^{4}$	$3.5{\cdot}10^{4}$	$9{\cdot}10^{5}$
Mevinphos	$5{\cdot}10^{4}$	$5{\cdot}10^{3}$	$2.5{\cdot}10^{3}$	$4.5{\cdot}10^{4}$	$5.5{\cdot}10^{5}$
Sarin	$1.2{\cdot}10^{7}$	$4.2{\cdot}10^{6}$	$4.8{\cdot}10^{7}$	$1.9{\cdot}10^{8}$	$2.5{\cdot}10^{8}$
Soman	$7.8{\cdot}10^{7}$	$1.5{\cdot}10^{7}$	$3{\cdot}10^{7}$	$3{\cdot}10^{8}$	$9{\cdot}10^{7}$
Aminostigmine	$5{\cdot}10^{6}$	$7.7{\cdot}10^{5}$	$1{\cdot}10^{5}$	$3.5{\cdot}10^{5}$	$1{\cdot}10^{3}$
Physostigmine	$8{\cdot}10^{6}$	$2{\cdot}10^{6}$	$1.2{\cdot}10^{6}$	$1.6{\cdot}10^{5}$	$1.1{\cdot}10^{4}$
Bizerine	$3.3{\cdot}10^{6}$	$9{\cdot}10^{5}$	$4{\cdot}10^{5}$	$8{\cdot}10^{5}$	$4{\cdot}10^{3}$
X-129	$2.6{\cdot}10^{9}$	$4{\cdot}10^{7}$	$6{\cdot}10^{6}$	$5{\cdot}10^{6}$	$2.4{\cdot}10^{3}$
Sevine	$2.5{\cdot}10^{4}$	$2.5{\cdot}10^{3}$	$1{\cdot}10^{3}$	$1{\cdot}10^{3}$	$4{\cdot}10^{2}$

The sensitivity of FChE is 70 times higher for dipterex, that of DDVP - 1500 times, phosphamidon - 160 times, dimethoat - 90 times, mevinphos - 100 times, sarin - 50 times than the sensitivity of BuChE. It is very unexpectedly and unordinary that FChE has a very small sensitivity to active carbamates.The sensitivity of FChE for physostigmine is 200, aminostigmine - is 700, bizerine- is 250, bis-quaternary carbamate X-129 - is 15 000 times lower than the sensitivity of BuChE.

The results obtained with study of kinetic behaviour of FChE and its sensitivity to OP and carbamates suggested that there might be a essential difference between the active sites of FChE and another types of cholinesterases. It is very important that with the help of a new purified FChE the separate bioidentification of OP (especially the organophosphorus pesticides) and carbamates may be carried out. The extremely low sensitivity of new purified enzyme to carbamates is particularly valuable for these purposes.

 ISBN: 978-81-928924-3-6

References

1. Silver. Ann. The biology of cholinesterases. Elsevier publish Company, 1974, 236.
2. Augustinsson. K.-B. The evolution of esterases in vertebrates Homologous Enzymes and Biochemical Evolution, Gordon and Breach, New York, 1968, 299 - 311.
3. Augustinsson, K.-B. Electrophoresis studies of blood plasma esterases. Acta Chem. Scand.1959, 13, 2, 571 – 592.
4. Pavlov. D., Feeding behavior and brain acetylcholinesterase activity in bream (*Abramisbrama*) as affected by DDVP. Comp. Biochem. Physiol.,1992,103C, 3, 563-568.
5. Kozlovskaya,V. et al. Cholinesterases of invertebrates. Rev. Environ. Contam. Toxicol.1993, 132, 117 - 142.
6. Ellman, G. et al. A new and rapid colorimetric determination of acetylcholinesterase activity.Biochem.Pharmacol.1961,7, 2, 88 - 95.
7. Lineweaver, H., Burk, D. J. The determination of enzyme dissociation constants.J.Amer.Chem. Soc. 1974,55, 6,658 - 666.

Chapter 7

A Lab-scale investigation into the potential polybrominateddiphenyl ethers and trace metals complexation in aqueous media

***Adegbenro P. Daso[1], Jonathan O. Okonkwo[2], Patricia B. C. Forbes[1] and Egmont R. Rohwer[1]**

[1]Department of Chemistry, Faculty of Natural and Agricultural Sciences, University of Pretoria, Lynwood Road, Private Bag X0028, Hatfield, Pretoria 0002, South Africa.

[2]Department of Environmental, Earth and Water Sciences, Faculty of Science, Tshwane University of Technology, 175 Nelson Mandela Drive, Arcadia Campus, Pretoria, South Africa.

***Corresponding author:** adegbenrop@yahoo.com

Abstract

Polybrominateddiphenyl ethers (PBDEs) are important industrial chemicals widely used as flame retardants in various consumer products, including computers, furniture, electronics, textiles, carpets, amongst others. In the recent past, there were concerns about the potential health risks associated with their bioaccumulation, toxicity, environmental persistence as well as their rapidly increasing levels in the environment. Incidentally, the possible interactions between PBDEs and other secondary contaminants, especially the transition elements in aquatic environment are yet to be thoroughly considered.Therefore, this study aims to investigate the potential ligand-metal interactions between certain PBDE congeners and some transition metals (Cobalt, Copper, Iron, Lead, Manganese and Zinc) using gas chromatography mass spectrometry (GC-MS), UV-visible spectrophotometry and Fourier Transform Infra-red spectroscopy (FTIR).

The preliminary investigation conducted using a 3-paired (spiked and unspiked) experimental set-up showed relative percentage losses of PBDE congeners possibly due to complexation with the spiked trace metals, which were up to 41% (BDE 183), 45% (BDE 183) and 75% (BE 100) in the three set-ups. Similarly, a significant shift from 2900 cm^{-1} to 1700 cm^{-1} in the FTIR spectra of PBDEs solution and PBDE solution spiked with trace metals suggested that there could be a significant interaction between PBDEs and the transition metals in aqueous media. Unraveling the underlying factors contributing to the potential complexation between PBDEs and transition metals is critical. This is because the complexation of PBDEs with environmentally relevant levels of trace metals could result in their non-detection or under-estimation in polluted aquatic environment. However, when the conditions become favourable, PBDEs, which acts as a ligand may be released from the complexes,

formed. Significantly, these ligand-metal interactions in aquatic environment could pose serious exposure problems to aquatic organisms and thus need to be considered for risk assessment of PBDEs and other similar organic pollutants.

Keywords

PBDEs, complexation, trace metal, aqueous media, GC-MS.

1. Introduction

Due to the strict precautionary measures required for a safe use of certain consumer products, some categories of industrial chemicals generally referred to as flame retardants are deliberately incorporated into these products to ensure that the required safety standards are met. Polybrominateddiphenyl ethers (PBDEs), which are important class of brominated flame retardants, have been widely used for this purpose due to their excellent flame retarding properties. In fact, up to 30% by composition of PBDEs may be incorporated into different components of the treated products such as computers, TV sets, furniture, carpets, amongst others (La Guardia, Hale & Harvey, 2006). Although, their use has been beneficial, there are still growing concerns related to their continued usage because of their high degree of environmental persistence, toxicity, potential to bioaccumulate in living tissues as well as their capability to undergo long-range atmospheric transport.

Because of their prolonged use, PBDEs have become ubiquitous and thus, have been detected in different environmental and biological matrices (Gauthier *et al.*, 2008; Peng *et al.*, 2009). Discharges from wastewater treatment plants (WWTPs) represent an important pathway of PBDEs and other emerging organic pollutants into the receiving environment (Daso, Fatoki & Odendaal, 2013; Song *et al.*, 2006). Incomplete removal of these organic pollutants during WWTP processes could result in significant transfer of PBDEs together with other secondary contaminants into the adjacent water bodies. While efforts have been made to assess the concentrations of PBDEs and other emerging organic pollutants in different aqueous matrices, including WWTP effluents, the potential influence of the presence of increasing levels of these secondary contaminants on the ultimate fate of PBDEs in these matrices has not been thoroughly investigated.

Several possible interactions between PBDEs and these secondary contaminants such as trace metals and certain anions could occur within the aqueous matrices. Structurally, PBDEs may act as potential ligands to a number of trace metals, especially the transition metals, resulting in the formation of ligand-metal complexes, and in addition, certain anions may also compete with PBDEs as potential ligands.

In this preliminary study, an attempt was made to investigate some of these possibilities using spectroscopic as well as chromatographic techniques. Specifically, the potential interactions between the commonly investigated PBDE congeners and some potentially harmful trace metals were investigated, although the scope of the study was streamlined to a few metals that showed good responses to certain analytical instruments employed during the study.

2. Materials and Methods:

2.1 Reagents:

Isooctane and individual trace element standards 1000 mg/L (Co, Cu, Fe, Mn and Zn) were purchased from Merck KGaA (Darmstadt, Germany). Individual PBDE standards (BDE

 ISBN: 978-81-928924-3-6

17, 47, 100, 99, 154, 153, 183 and 209) produced by Cambridge Isotopes Inc. (MA, USA) were supplied locally by Industrial Analytical (Pty) (Midrand, South Africa). SPE cartridges (Supelclean™ ENVI™-18) and Chromasolv® for HPLC organic solvents (n-hexane, acetone, dichloromethane (DCM), methanol) were purchased from Sigma-Aldrich (Aston Manor, South Africa).

2.2 Sample collection:

For the purpose of determining the actual concentrations of the trace elements in the effluent-receiving water bodies, surface water samples were collected from two major rivers, namely Juskei and Vaal that are notable for effluent discharges within the Gauteng region. The samples were collected and preserved using standard protocols for surface water sampling between October and November 2013. The samples were stored in the refrigerator until analysis.

2.3 Procedure for Atomic Absorption Spectrophotometry (AAS) for trace metals determination:

About 100 mL each of river water samples (*N*=2) was carefully measured into clean beakers. A total of 10 mL conc. HNO_3 was added to each sample. The samples were placed on hotplate for digestion for about 3 hours or until a clear yellowish solution was obtained. Thereafter, the digested samples were allowed to cool to room temperature and were subsequently filtered and made up to 100 mark in volumetric flasks using deionized water. A laboratory blank was similarly prepared and analysed together with the samples. The surface water samples were prepared in triplicates and analysed using Spectra AA 220FS atomic absorption spectrometer.

2.4 Procedure for Gas Chromatography- Mass Spectrometry (GC-MS) for PBDEs analysis:

Two experimental set-ups were designed for the investigation of potential interactions between PBDEs and certain trace metals using the GC-MS technique. In both cases, ultra-pure water obtained from a Labosfar™ D1 4 Ultrapure water system (Barsbüttel, Germany) was used. Firstly, a set of triplicate samples were spiked with known amount of PBDEs (250 µL of 48 ppb for tri-hepta-BDEs and 480 ppb for BDE 209), while the trace metals (Co, Cu, Fe, Mn and Zn) concentrations in the solution were adjusted to 0.01 ppm similar to the concentrations obtained for most of the metals in the analysed river water samples. The other set of triplicate samples were similarly treated but were not spiked with the trace metals. These set-ups were allowed to equilibrate for about 3 hours prior to their extraction with solid-phase extraction (SPE) technique. The SPE cartridges were initially pre-conditioned using 6 mL each of n-hexane, DCM, methanol and deionized water following this sequence. Thereafter, the samples were extracted at a flow rate of approximately 2 drops per second. Upon the completion of the extraction procedure, the sample containers were thoroughly rinsed with the 10 mL of deionized water, which was subsequently transferred into their respective cartridges. The cartridges were allowed to dry at high pressure for 30 – 45 min. Finally, the target analytes were eluted under gravity with 6 mL of freshly prepared n-hexane: DCM (1:1, v/v). The extracts obtained were spiked with 500 µL of isooctane and were blown to near dryness under a gentle stream of nitrogen gas. These were later reconstituted with 250 µL of 100 ppb pentachloronitrobenzene employed as internal standard prior to the GC-MS

analysis using an Ultra-trace 2010 Shimadzu GC equipped with QP 2010 Ultra mass spectrometer operated in EI mode.The chromatographic separation of these compounds was achieved using ZB-5 MS (15 m, 0.25 mm i.d., 0.25 µm film thickness) capillary column. The oven temperature programming was as follows: 90°C held for 1.0 min., ramped @ 40°C/min. to 200°C, ramped @25°C/min to 250°C, ramped @ 7.5°C/min to 310°C held for 5 min.

2.5 Procedure for UV-visible and Fourier Transform Infra-red (FTIR) spectrophotometric determination:

To determine the absorption maxima for the trace metals being investigated, 100 ppm of individual trace metal solution was prepared. These standard solutions were subjected to UV-visible spectroscopy scanning between 1000 and 250 nm to obtain the absorption maxima for the respective trace elements employing Lambda 25 UV-visible spectrophotometer (Perkin Elmer, England). In this case, only trace elements with coloured solutions, except for Fe were successfully determined. The mean absorption maxima for the solutions of these metals were 517.08 and 815.96 nm for Co and Cu, respectively.

Similarly, the UV-visible spectra for standard solutions of BDE 47 (50 ppb), PBDE mix (mixture of 8 congeners, 48 ppb for tri-hepta-BDEs and 480 ppb for BDE 209) as well as for isooctane and the blank (mixture of deionized water and isooctane) were obtained. Furthermore, the spectra for the mixture of standard solutions of the trace metals (3 mL of 0.01 ppm for all the metals) and PBDE mix (mixture of 8 congeners, 1 mL of 48 ppb for tri-hepta-BDEs and 480 ppb for BDE 209) after vigorous agitation of the mixture was obtained. Similar tests were performed for the mixtures of BDE 47 (1 mL of 50 ppb) and Co (3 mL of 100 ppm) as well as BDE 47 (1 mL of 50 ppb) and Cu (3 mL of 100 ppm). Finally, the effect of contact time was investigated for the mixture of BDE 47 and Co where UV-visible spectra were obtained for these set-ups immediately after agitation and after 15 and 30 minutes of contact between the two reacting species.

FTIR technique was employed to provide qualitative information on the potential interactions between the trace elements and PBDEs using a Spectrum RX1 FTIR system (Perkin Elmer, England) equipped with a specialized unit for the analysis of aqueous samples. Spectra were obtained for the individual standard solutions of the trace metals, PBDE mix as well as the mixtures of these reacting species.

3. Results and Discussion:

3.1 Trace metals profiles of river water samples:

To ascertain the appropriate spiking concentrations of the trace metals being investigated in this study, an initial profiling of the river water samples collected from the Juskei and Vaal Rivers was undertaken. The results obtained from this assessment are presented in Table 1, where the calibration concentrations range from 1 to 5 ppm for all the trace metals.

Table 1: Trace metals concentrations (ppm) of water samples collected from the Vaal and Juskei Rivers.

Sample	Fe	Cu	Co	Mn	Zn
VA1	>5.000	0.017	0.002	0.220	0.071

VA2	4.398	0.008	0.002	0.242	0.026
VA3	>5.000	0.004	0.01	0.234	0.026
Mean	**-**	**0.010**	**0.005**	**0.232**	**0.041**
SD	**-**	**0.007**	**0.005**	**0.011**	**0.026**
JUK1	0.077	0.006	0.010	0.035	0.028
JUK2	0.063	0.006	0.005	0.039	0.018
JUK3	0.171	0.004	0.007	0.033	0.019
MEAN	**0.104**	**0.005**	**0.007**	**0.036**	**0.022**
SD	**0.059**	**0.001**	**0.003**	**0.003**	**0.005**

The trace metals profiles generally showed different results for all the investigated metals. This probably reflects their natural abundances or possibly indicating the potential anthropogenic contributions of these metals into the receiving water bodies. Unfortunately, most of the spectrometric techniques employed in this study, especially the UV-visible spectrometry was not sensitive enough to detect these metals at the levels found in the river water samples. Hence, relatively high levels of these metals were often employed during the investigation. For the purpose of uniformity, a spiking concentration of 0.01 ppm was prepared for all the trace metals and was employed for further investigation on the potential interaction between PBDEs and the trace metals using GC-MS and FTIR techniques.

3.2 Gas chromatography-mass spectrometry:

To investigate the potential interactions between PBDEs and certain trace metals, a 3-paired set-up comprising of spiked and unspiked samples were employed. As previously mentioned, set-ups (1-3) were spiked with standard solutions of the reacting species, while set-ups (4-6) were only spiked with PBDEs standards. This arrangement is necessary to establish the potential influences of the spiked trace metals on PBDEs and to ultimately indicate if there is any sort of interactions between the two species. The results obtained from the GC-MS analyses of the experimental set-ups are shown in Table 2 below.

Considering the poor recoveries of most of the PBDE congeners investigated in this study, it is reasonable to conclude that this technique would not provide appropriate quantitative information required to fully understand the potential interactions between PBDEs and the trace metals being investigated. Hence, the choice of electrochemical techniques such as anodic stripping voltammetry, which has the capabilities to detect trace levels of trace metals, would be of immense advantage in fully understanding the possible interactions between PBDEs and the investigated trace metals. Generally, there are potential losses as much as 75% observed during these experiments, however, it would be grossly misleading to attribute these losses to possible complexation between PBDEs and the trace metals.

Table 2: The percent losses of PBDE congeners in the 3-paired experimental set-ups.

Target compound	Target and reference ions	Paired set-up 1			Paired set-up 2			Paired set-up 3		
		1	**4**	**% Loss**	**2**	**5**	**% Loss**	**3**	**6**	**% Loss**
BDE 17	**246**, 326, 404	18.21	20.47	11.01	19.10	19.67	2.94	20.75	19.67	-5.49
BDE 47	**326**, 404, 564	25.92	38.20	32.16	33.80	38.32	11.81	35.60	44.59	20.16
BDE 100	**404**, 406, 566	0.74	2.20	66.35	1.37	1.79	23.45	0.96	2.65	63.85
BDE 99	**404**, 406, 566	4.15	3.46	-20.00	2.67	3.83	30.29	2.68	4.25	36.99
BDE 154	**484**, 406, 566	6.58	18.57	64.59	10.79	9.22	-16.93	11.48	9.87	-16.33
BDE 153	**484**, 486, 645	4.24	16.90	74.93	8.51	9.06	6.10	9.57	7.61	-25.65
BDE 183	**561**, 564, 721	2.36	5.39	56.21	2.31	4.21	45.13	2.30	3.88	40.79

(**Bold** – target ions)

Other potential contributors to these losses may include adsorption to sample container walls, volatilization and poor retention of PBDEs as well as the formation of weak ligand-metal complexes, which easily dissociate during the SPE process. From these foregoing, it is clear that the use of several complementing techniques is required to be able understand the nature of the interactions occurring between PBDEs and the investigated trace metals.

3.3 UV-visible and FTIR spectrometry:

Presently, there is little or no information on the formation chemistry of PBDEs and trace metals. However, spectroscopic evidence of interactions between certain trace metals and natural organic matter has been reported (Hoffmann, Mikutta & Kretzschmar, 2013). In this study, we explored the coloured solutions of both Co (pink) and Cu (blue) to conduct UV-visible experiments so as to establish any possible interaction between the ligand (PBDEs) and these metals. A major setback with this technique is its extremely low sensitivity, which requires relatively high levels of the reacting species to be employed.

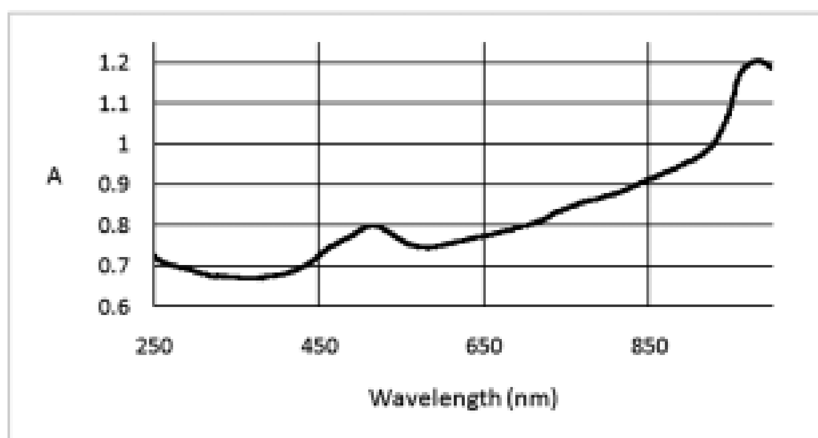


Fig. 1: UV-visible spectrum showing the absorption maximum of Co at 525 nm.

The levels of the reacting species, especially the trace metals used in these experiments were several orders of magnitude higher than those present in the analysed river water samples. This particularly makes it difficult to mimic the natural conditions of the investigated water bodies. Consequently, it was extremely difficult to ascertain the possibility of potential interactions between the reacting species. This effect was even more pronounced when equal volumes of the reacting species were mixed together and subjected to UV-visible spectroscopy.

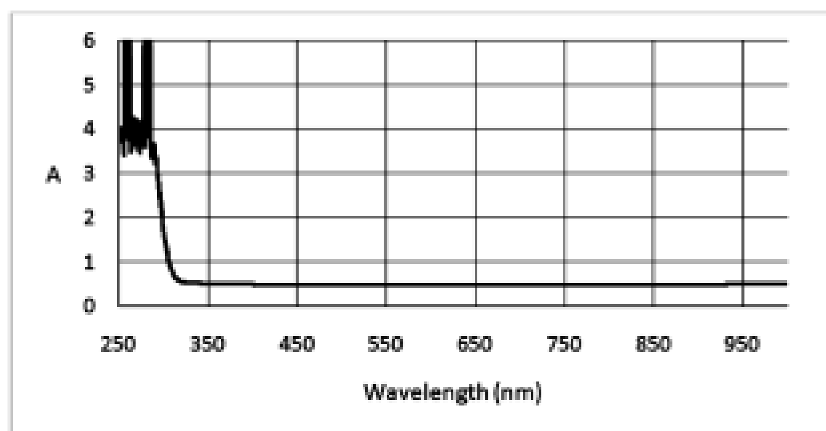


Fig. 2: UV-visible spectrum obtained for a mixture of Co and PBDEs.

As shown in Figure 1, the absorption maximum of Co at 525 nm was visibly noticed when 2 mL of 48 ppb PBDE mix was mixed with 2 mL of 100 ppm of Co. However, when the proportion of the PBDE mix was significantly reduced by 100 fold (i.e. 20 µL of 48 ppb PBDE mix and 3980 µL of 10 ppm), the absorption maxima as shown in Figure 2 completely disappeared. This shows that some sorts of interactions between the two species have occurred. Incidentally, a similar pattern was not observed when similar mixed solutions of Cu and BDE 47 were analysed. This clearly suggests that there are potential interactions between the trace metals, especially Co and PBDEs; however, these can only be observed when low concentrations of both species are involved.

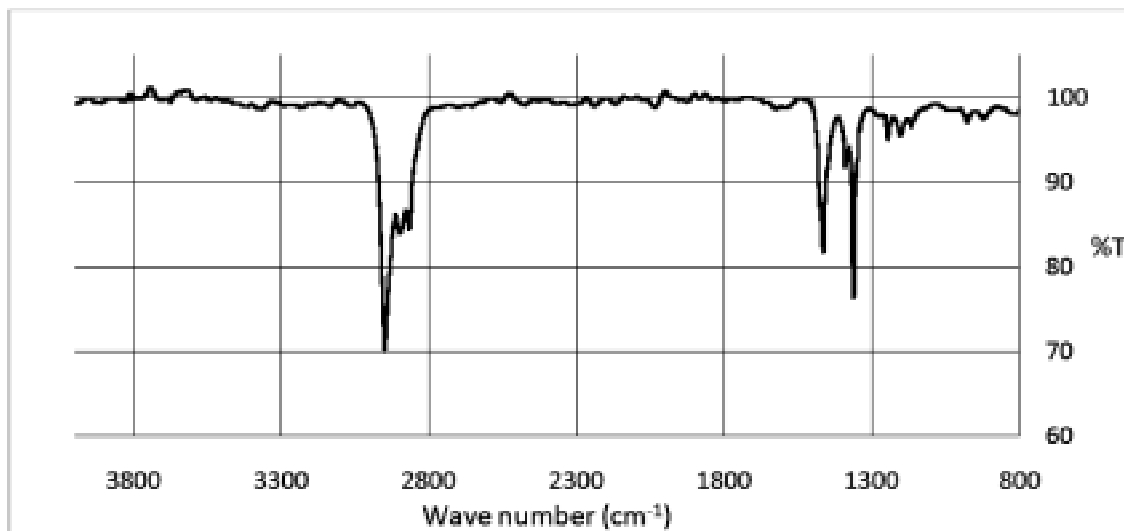


Fig. 3: FTIR spectrum obtained for PBDEs.

The FTIR analysis showed interesting results. First, the spectra obtained for the mixtures of PBDE congeners as shown in Figure 3, indicated three prominent peaks at approximately 2900, 1450 and 1400 cm^{-1} probably indicating the C-H vibrational and stretching bond frequencies of the isooctane in which the PBDEs were suspended. Incidentally, the relative frequencies of these prominent peaks changed significantly when both PBDEs and trace metals solutions were mixed together.

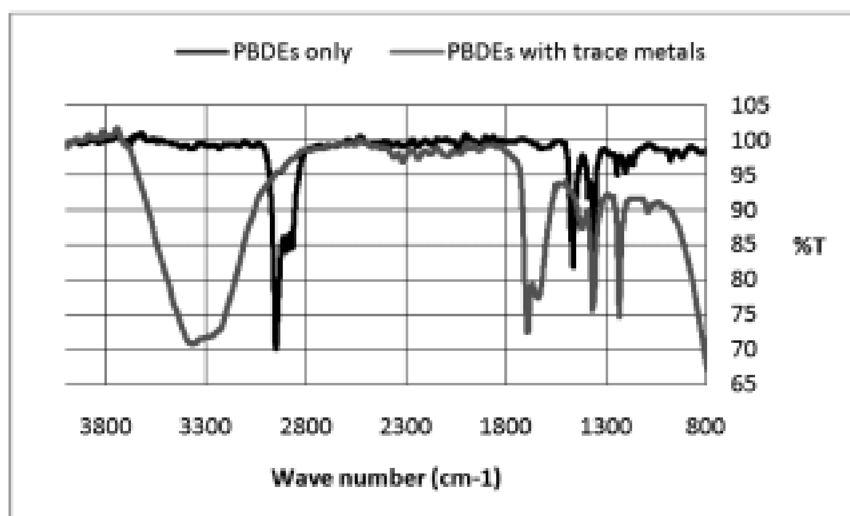


Fig. 4: Spectra overlay showing the shifts indicating potential interactions between PBDEs and trace metals

As shown in Figure 4, while the peaks at 1450 and 1400 cm^{-1} experienced minor shifts in frequencies, the peak at the 2900 cm^{-1} had a major shift from this wave number to around 1700 cm^{-1}. This major shift is probably indicative of important interactions taking place between

the two reacting species. While it is difficult to identify the particular species in the mixtures thatis responsible for these interactions, future efforts would be directed towards unravelling the present absurdity with respect to the potential interactions between PBDEs and certain trace metals investigated in this study.

4. Conclusion:

The findings from these preliminary studies clearly show that there are potential interactions between PBDEs and certain trace metals investigated. It was observed that employing higher concentrations as well as volume of the reacting species made the investigation of their potential to form a complex difficult to determine when UV-visible spectrometric technique was employed. Similarly, the SPE procedures followed by GC-MS technique employed in this study could not provide sufficient information on the potential interactions between the reacting species due to the possible losses from adsorption onto the container walls. Interestingly, the FTIR is the only promising technique amongst those employed in this study that produces sufficient information on the interactions between the reacting species. More studies involving the use of complementary techniques such as anodic stripping voltammetry need to be conducted to fully understand the chemistry behind the interactions taking place between the two reacting species.

References

1. DASO, A. P., FATOKI, O. S. & ODENDAAL, J. P. Occurrence of polybrominated diphenyl ethers (PBDEs) and 2, 2', 4, 4', 5, 5'-hexabromobiphenyl (BB-153) in water samples from the Diep River, Cape Town, South Africa. *Environmental Science and Pollution Research*:2013.1-9.
2. GAUTHIER, L. T., HEBERT, C. E., WESELOH, D. C. & LETCHER, R. J. Dramatic changes in the temporal trends of polybrominated diphenyl ethers (PBDEs) in herring gull eggs from the Laurentian Great Lakes: 1982–2006. *Environmental Science & Technology,* 2008. 42(5):1524-1530.
3. HOFFMANN, M., MIKUTTA, C. & KRETZSCHMAR, R. Arsenite Binding to Natural Organic Matter: Spectroscopic Evidence for Ligand Exchange and Ternary Complex Formation. *Environmental Science & Technology,* 2013.47(21):12165-12173.
4. LA GUARDIA, M. J., HALE, R. C. & HARVEY, E. Detailed polybrominated diphenyl ether (PBDE) congener composition of the widely used penta-, octa-, and deca-PBDE technical flame-retardant mixtures. *Environmental Science & Technology,* 2006.40(20):6247-6254.
5. PENG, X., TANG, C., YU, Y., TAN, J., HUANG, Q., WU, J., et al. Concentrations, transport, fate, and releases of polybrominated diphenyl ethers in sewage treatment plants in the Pearl River Delta, South China. *Environment International,* 2009. 35(2):303-309.
6. SONG, M., CHU, S., LETCHER, R. J. & SETH, R. Fate, partitioning, and mass loading of polybrominated diphenyl ethers (PBDEs) during the treatment processing of municipal sewage. *Environmental Science & Technology,* 2006.40(20):6241-6246.

Chapter 8

Diesel Toxicity and Soil Remediation

***Agamuthu, P.and Dadrasnia, A**.
Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603
Kuala Lumpur, Malaysia.
***Corresponding author:**agamuthu@um.edu.my

Abstract

Hydrocarbons from different sources constitute major soil contaminations across the globe. Modern industrial society is built and ruled by petroleum hydrocarbons and large areas of water and soil are contaminated with toxic chemicals and oil-derived compounds. More than 1.8 million tonnes of crude oil produced are released into the environment annually. Public, government and the industry now recognized the potential risk of organic compounds such as total petroleum hydrocarbons (TPHs), polycyclic aromatic hydrocarbons (PAHs)to environment, human and animal health. Sustainable technologies need to be developed and promoted for environmental management that would apply to a wide range of terrestrial habitats and aquatic environment. Due to cleanup techniques of petroleum-contamination problems, it has been demonstrated that it required developing remediation technologies that are quick, feasible and economical in a wide range of polluted sites. Therefore, many studies have reported bio/phytoremediation, and scientific literature has displayed the advanced mechanisms of different remediation techniques. The selection of methods depends on contaminated sites' characteristics, costs, time constrain and requirements. Therefore, successful remediation of contaminated sites will depend on the selection of remediation techniques which is a very important step, and adjustment and design of remediation techniques, as explained in the case study here.

Keywords:
Environmental pollution, Organic compound, Remediation techniques

1. Introduction:

1.1. Introduction to soil contamination:

The global environment is under great stress due to industrialization and urbanization, as well as population pressure on the limited natural resources. The problems are compounded by drastic changes that have been taking place in the lifestyle and habits of people. The nature and the importance of the problems are always changing, bringing new challenges and need for developing newer and more suitable technologies. Soil contamination is caused by industrial activity, chemicals or disposal of wastes or other natural sources released (into any

environmental medium) by human beings. The most common chemicals involved are aromatic hydrocarbons, pesticides, solvents and many heavy metals such as lead (Sharma, 2012). Petroleum is essential to the current global network economy, without it, our economic order would cease to function, bringing disaster to many populations. Polycyclic aromatic hydrocarbon (PAHs) as the most important source of energy for daily life and the high industrial usage are commonly found as organic pollutant in the environment (Collins, 2007). The unintended release of hydrocarbons into the environment can negatively impact human and animal health, and change the characteristics of soils impacting the plant populations they can support. Hydrocarbons of diesel origin can become embedded in the matrix of soil particles.

To appreciate the magnitude of unintended hydrocarbon release let us look at some global statistics. In 2003, the world consumption of petroleum was over 63.5 million barrels per day (Jain et al., 2011). Table 1 shows the number of oil spills in the world. Sonawdekar (2012) reported that the amount of natural crude oil spill was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year.

Table 1. Annual number of oil spills

Year	< 700 tonnes oil spills	> 700 tonnes oil spills
2007	13	4
2008	8	1
2009	7	1
2000s Total	**149**	**33**
Average for decade	**14.9**	**3.3**
2010	4	4
2011	4	1
2010s Total	**8**	**5**
Average	**4**	**2.5**

(ITOPF, 2011)

1.2. Chemical properties of hydrocarbon

When the suitability of a cleanup approach is to be evaluated, the biodegradability and composition of the petroleum hydrocarbon pollutant is the most important consideration (Okoh 2006). Compositional heterogeneity among different crude oils and refined products influenced the overall rate of biodegradation of both the oil and its component fractions. Biodegradability is essentially impacted by the composition of the oil pollutant (Jain et al. 2011). For example, kerosene can be completely degraded, under suitable conditions, but in the case of heavy asphaltic-naphthenic crude oils a maximum of about 11% is biodegradable even under suitable conditions (Okoh and Trejo-Hernandez 2006). Okoh et al., (2003) indicated that between 8% and 30% of the crude oil was degraded within 15 days in polluted soil by mixing bacterial consortium.

 ISBN: 978-81-928924-3-6

2. Remediation methods:

Remediation of contaminated sites can be achieved through physical and chemical techniques such as disposal in landfill, incineration, use of chemical oxidants and biological processes (Ayotamuno et al., 2009). Conventional remediation technologies are time consuming, expensive and environmentally divesting. The traditional treatment, physical and chemical methods may not remove and degrade the oil thoroughly. Hence, it is unavoidable to use an environmentally friendly technology and low cost method to remediate polluted soils, specifically in developing countries. Compared to physico-chemical methods, biological processes are thought to be of low environmental risk and low cost but in some cases, longer time is required. These recent systems are based on the stimulation of aerobic bacteria populations to degrade contaminants, which is done by increasing oxygen flux and adding nutrients to the contaminated zone. All remediation techniques are used to remove pollutants either in situ (in place) or ex situ (other sites for treatment). According to the Office of Technology Assessment, conventional methods typically recover no more than 15% of the oil after a major oil spill (Zahed et al., 2006). Some common physicochemical methods for remediation of soil such as, soil washing with solvents and water, Soil vapor extraction, Solidification/stabilization and biological technologies (bioremediation and phytoremediation).

The selection of remediation method and the determination of the final remediation standard are always affected by factors such as site-specific including local conditions and sensitivities, available timeframe and potential risk. Therefore, it is appropriate to mention the available technologies at this point, in order to set the discussions of the specifically biotechnological methods to come.

2.1. Current clean up techniques:

The currently available remediation techniques can be divided into five categories:

- Biological; this method involves of the mineralization or transformation of contaminants to more toxic but less mobile or more mobile but less toxic forms. The main advantage of biological technique is to destroy a wide range of organic compounds.
- Chemical; in this method toxic compounds are fixed, destroyed, or neutralized by chemical reaction. In this technique toxic substances can be chemically converted to less or more biologically available.
- Physical; in this method the removal of contaminated materials is achieved often by excavation or further treatment or disposal.
- Solidification/vitrification; this method as used in both the physical and chemical processes to reduce potentially adverse impacts on the environment resulting from the disposal of hazardous and mixed wastes. The stabilization technique is used to chemically reduce the soluble in hazardous wastes by converting the contaminants to reduced solubility (Silva et al., 2007).
- Thermal; in this method using heat treatment such as gasification, incineration, volatilization or pyrolysis processes. The advantage of this technique is that the contaminants are most effectively destroyed. On the other hand, this method is of very high energy cost, and the approach is unsuitable for most toxic elements.

Table 2 shows different treatment methods which are employed for the remediation of soil. Most remediation technologies are site specific and the selection of appropriate technologies is often a difficult, but extremely important step in the successful remediation of a contaminated site (Khan et al., 2004). Therefore, the successful treatment of a contaminated site depends on proper selection, design, and adjustment of the remediation technology's operations based on the properties of the contaminants and soils, and on the performance of the system.

Table 2. Treatment techniques used for soil remediation

	Techniques	References
§	Physical treatments	
-	Soil washing	(López-Vizcaíno et al., 2012)
-	Stabilization	(Fleri and Whetstone, 2007)
§	Chemical treatments	
-	Oxidation	(Lemaire et al., 2011)
-	Photo degradation	(Dadrasnia and Agamuthu,2013b)
-	Peroxide remediation	(Qi et al., 2004)
§	Biological treatment	
-	Bioremediation	(Dadrasnia and Agamuthu, 2013a)
-	Phytoremediation(Agamuthu et al., 2010)	

2.2. Bioremediation of contaminated land

Biological methods are those techniques which depend on the microbial activity to break down and mineralize of contaminates to less to toxic form (Sayara, 2010). Bioremediation describes the process of contaminant degradation in the environment by biological methods using the metabolic potential of microorganisms to degrade a wide variety of organic compounds (Kumar et al., 2011). Remediation of petroleum-contaminated systems can be achieved by biological or physicochemical methods. However, the negative consequences of the physicochemical approach are currently directing greater attention to the exploitation of the biological alternatives (Okoh, 2006).

The advantages of the bioremediation systems over the other technologies have been summarized. Furthermore, it is a non-invasive technique, leaving the ecosystem intact (Perelo, 2010). Bioremediation can deal with lower concentration of contaminants where the cleanup by physical or chemical methods would not be feasible. Besides cost effectiveness, it is a permanent solution, which may lead to complete mineralization of the pollutants (Perelo, 2010). Bioremediation can deal with lower concentration of contaminants where the cleanup by physical or chemical methods would not be feasible.Bioremediation is most suited to organic compounds, but it can be effective in the remediation of certain inorganic substances

ISBN: 978-81-928924-3-6

too (radionuclides and metals). Indeed, the result produced could be a very effective functional remediation, under the right conditions. A process typically used to degrade solid waste materials, has also recently been studied as a remediation technology for PAH contaminated soils (Gan et al. 2009). This technique involves combining contaminated soil with nonhazardous organic amendants such as agricultural wastes or manure (Kumar et al. 2011). Composting strategy is an aerobic process, based on mixing components of composting with the contaminated soil, as the compost matures the pollutants are degraded by the active microflora within the mixture (Table 3). Dadrasnia and Agamuthu (2013a) recorded 85% loss of oil in soil contaminated with diesel oil and amended with soybean cake for a period of 84 days. Haderlein et al., (2006) studied the effects of composting to soil by the addition of maple leaves and alfalfa during the mineralization of pyrene and benzopyrene and reported that neither composting nor the addition of compost had any effect on benzopyrene mineralization. In contrast, the pyrene mineralization rate increased dramatically with the amount of time that the soil had been composted (more than 60% mineralization after 20 days). In a related study, solid culture with a small amount of low-quality raw coffee beans was used for total petroleum hydrocarbon removal from a weathered and polluted soil (Adriana et al. 2007). Amendment of soil contaminated by heavy mineral oil using sawdust, hay and compost was reported by Lee et al., (2008) that after 105 days of experiment the heavy mineral oil was reduced between 18 - 40% from the initial level of contamination of 7490 mg hydrocarbon kg^{-1}, whereas the level of hydrocarbon reduction in non-amended soil was just 9%.

2.3. Phytoremediation (phytotechnology)

Phytoremediation, a green technology, is quite a novel technique which uses plants to remediate contaminated sites such as soil, sediment, surface water and groundwater (Kim et al. 2007). Phytoremediation is relatively easy to implement and is cost-effective at minimal maintenance overheads, and as long as the impacted site can support plant growth, a remediation scheme can be used anywhere (Couto et al. 2012). Phytoremediation appears effective, inexpensive and attractive because in contrast to most other remediation technologies, it is not invasive and, in principle, delivers intact and biologically active soil (Wenzel 2008). This technique can be used to remediate heavy metals, radioactive materials, and petroleum hydrocarbon. It might be because this method is very slow and takes time (some time more than 10 years), which makes it difficult to evaluate in the early state.

Table 3. Overview on remediation using composting materials

Composting material	Contaminated media	Reference
banana skin, brewery spent grain, and spent mushroom compost	Lubricating oil contaminated soil	(Abioye et al. 2012)
Cow dung, poultry manure and pig wastes	soils spiked with waste-lubricating oil	(Adesodun and Mbagwu 2008)

raw coffee beans	Petroleum hydrocarbon contaminated soil	(Adriana et al. 2007)
tea leaves, potato skin and soy cake	Diesel fuel contaminated soil	(Dadrasnia and Agamuthu 2013b)
chicken droppings	crude oil contaminated soil	(Ijah et al. 2008)

Furthermore, phytoremediation is limited to contamination within the depth of the rhizosphere or the depth of influence from evapotranspiration, depending on the most important removal mechanisms in the specific phytoremediation application. Microbe-assisted phytoremediation, including rhizoremediation, appears to be particularly effective for removal and/or degradation of organic contaminants from impacted soils particularly when used in conjunction with appropriate agronomic techniques(Gerhardt et al. 2009). Major drawbacks of phytoremediation include the fact that the detoxification of organic pollutants is often slow and if decomposition is not complete, toxic compounds may accumulate in plant tissue and be released into the environment or enter food-chains (Perelo 2010).

2.4. Interaction between Plants and Microorganisms

The efficiency of phytoremediation depends mostly on the establishment of robust plant-microbe interactions; however, little is known about how these interactions are influenced by petroleum pollution (Nie et al. 2011). Indeed, interaction between bacteria and plant will affect plant growth either directly or indirectly. Plants, through their 'rhizosphere', could support the hydrocarbon-degrading microbes that assist in phytoremediation in the root zone (Nie et al. 2011). For example, root activities in alfalfa and perennial ryegrass increase the number of rhizobacteria capable of petroleum degradation in the soil (Nie et al. 2011). Then microbes can enhance soil nutrient availability to the plants. Petroleum hydrocarbon is identified as harmful not only for plant growth but also to the microbe's community. In order to better understand the interactions of petroleum hydrocarbons on microbe-plant there is a need to improve the feasibility and sustainability of phytoremediation. Since in phytoremediation process the time requirements are sometimes longer compare than some traditional techniques such as incineration, it is not suitable for rapid treatments.

2.5. Cost of remediation

Costs data for remediation sites are limited. This section summarizes the available data on bioremediation projects including in-situ and ex-situ of soil remediation (Table 4). In average the cost of enhanced remediation ranged from 30 US dollar to 100 US dollars per cubic meter of soil. Many factors can affect the cost such as soil type, quantity and types of contaminant and amendments used (FRTR 2012). In situ *bioremediation* techniques often *cost* less compared to other remedial options.

2.6. Stable isotopes: a tool to monitor biodegradation process

Compound specific isotope analysis (CSIA) is an analytical method that measures the ratios of naturally occurring stable isotopic ratios in environmental samples (EMD 2011). CSIA is a new approach in environmental investigation settings. Isotopic analysis is used in

 ISBN: 978-81-928924-3-6

petroleum exploration and geology. All contaminants made of various elements (multiple) and atoms change in isotopic ratios which lead to breaking of bonds between atoms. During the biodegradation of a compound, the chemical process in both biological and abiotic reactions caused changes in the isotopic ratios in compounds and CSIA is used to measure these changes. CSIA can be used to gain information, make decisions about monitoring, and select remediation options.

Table 4. Comparison of remediation treatment costs

Treatment	Approximate cost (US$/tonne soil)
Biological	5 – 266
Chemical	19 - 940
Physical	31 - 266
Solidification/stabilization	27 - 268
Thermal	47 - 1175

Source (Asha et al. 2010).

3. Case studies on bio/phytoremediation of diesel fuel contaminated soil in Malaysia

There are many industrial sites in Malaysia which has the potential to be contaminated and it is going to be a significant issue in Malaysia. Until recently, this type of pollution received very little attention in Malaysia. A case study was carried out to evaluate the efficiency of organic wastes (biowastes) as supplementations for remediation of diesel fuel contaminated soil in Malaysia. Three organic wastes [tea leaf (TL), Soybean cake (SC) and Potato skin (PS)] and two economically viable plants (*Dracaena reflexa* and *Podocarpus polystachyus*) were utilized to evaluate the biodegradation of diesel fuel in soil contaminated with different concentrations of oil.

For biodegradation studies, soils were treated with 20%, 15%, 10% and 5% (w/w) diesel fuel and amended with 10% and 5% TL, SC and PS. Completely randomized design was used for a period of 126 days under laboratory condition. At the end of 126 days, soil polluted with 20% diesel oil and amended with 5% TL recorded the lowest percentage of oil degradation (14.5%) and diesel utilizing bacteria at 30×10^5 colony-forming units (CFU) per gram of soil. The highest rate of biodegradation (95%) was recorded in soil polluted with 5% diesel oil and amended with 10% SC with the count of diesel utilizing bacteria at 210×10^5 CFU/g. First order kinetic showed that soil amended with SC had the highest rate of oil degradation and illustrates the least half-life for all the diesel fuel concentrations. Bioremediation of diesel fuel contaminated soil with biomass amendments was monitored for a period of one year under natural condition. Result indicates complete biodegradation of C_8 to C_{16} and remarkable biodegradation of C_{16} to C_{22} hydrocarbon fractions in contaminated soil amended with SC. SC amendment enhanced the biodegradation of diesel fuel by 95%. The reason for the high potential by SC might be due to its high nutrient level (especially N) compared to other amendments utilized in this study. The results obtained demonstrated the potential of organic wastes for oil bioremediation in the order SC > PS > TL.

In phytoremediation study, contamination of soil with 2.5% and 1% diesel fuel and amended with 5% of the three different organic residues was monitored for a period of 270 days under laboratory and natural conditions. About 98.8%, 90.3% and 19% oil loss was recorded in soil amended with SC, polluted with 1%, 2.5% and 5% diesel oil planted with *D. reflexa*, respectively. However, diesel contaminated soil with *Dracaena* but without organic wastes recorded 62%, 52.4% and 8.5% for 1%, 2.5% and 5% contamination, respectively under laboratory condition. Also 91%, 84% and 13.8% oil loss was recorded in soil amended with SC, polluted with 1%, 2.5% and 5% diesel oil with *P. polystachyus*, respectively. The remediation process was influenced by oil concentration and organic biomass added. However, *D. reflexa and P. polystachyus* root did not accumulate hydrocarbons from the soil, thus indicating that the mechanism of the oil degradation was via phytovolatilization or rhizodegradation. The results of these studies illustrated the potential of SC and the two plants (*D. reflexa and P. polystachyus*) as a good option for enhanced remediation of hydrocarbon-contaminated soil (Dadrasnia and Agamuthu, 2013a,b).

4. Conclusion:

Contamination of soil environment by hydrocarbons (mostly petroleum hydrocarbons) is becoming prevalent across the globe. This is due to heavy dependence on petroleum as a major source of energy throughout the world. Many techniques of remediation of such contaminated soil have been developed, such as chemical degradation, photodegradation. However, most of these methods have some drawbacks in completely remediating hydrocarbon contaminated soil. Some of these methods leave behind residue compounds which are more toxic to the environment than the parent compounds. One major drawback of bioremediation of hydrocarbon contaminated soil is that many bioremediation experiments that proved to be effective under laboratory condition usually do not produce the same results under field conditions due to variation in number of factors such as weather conditions. Further research need to be carried out in this area in order to harness the full potential of bioremediation for effective remediation of hydrocarbon contaminated soil under field conditions.

References

1. Abioye O.P., Agamuthu P., Abdul Aziz A.R. Biodegradation of Used Motor Oil in Soil Using Organic Waste Amendments. *Biotechnology Research International* 2012:8.
2. Adesodun J.K., Mbagwu J.S.C. Biodegradation of waste-lubricating petroleum oil in a tropical alfisol as mediated by animal droppings. *Bioresource Technology*2008, 99 (13):5659-5665.
3. Adriana R., Graciano C., Norma R., Ma.Dolores D., Refugio R. Solid culture amended with small amounts of raw coffee beans for the removal of petroleum hydrocarbon from weathered contaminated soil. *International Biodeterioration andBiodegradation*2007, 60:35 - 39.
4. Agamuthu, P., Abioye, O. P. and Aziz, A. A. Phytoremediation of soil contaminated with used lubricating oil using *Jatropha curcas*. *Journal of Hazardous Materials,* 2010, 179(1-3): 891-894.
5. Asha A.J., Sanjeev K. S., Ackmez M. A comprehensive overview of elements in bioremediation. *Rev Environ Sci Biotechnol* 2010, 9:215–288.
6. Ayotamuno, J., Kogbara, R. and Agoro, O. Biostimulation supplemented with phytoremediation in the reclamation of a petroleum contaminated soil. *World Journal of Microbiology and Biotechnology,* 2009, 25(9): 1567-1572.

 ISBN: 978-81-928924-3-6

7. Collins, C. D. Implementing Phytoremediation of Petroleum Hydrocarbons. In N. Willey (Ed.), *phytoremediation: methods and reviews,* Totowa, NJ: Wiley- Blackwell, 2007, 23:1-512.
8. Couto M., Pinto D., Basto M.C.P, Vasconcelos T.S.D. Role of natural attenuation, phytoremediation and hybrid technologies in the remediation of a refinery soil with old/ recent petroleum hydrocarbons contamination. *Environmental Technology*:2012, 1-8.
9. Dadrasnia, A., and Agamuthu, P. Dynamics of diesel fuel degradation in contaminated soil using organic wastes. *International Journal of Environmental Science and Technology,* 2013a,*10*(4), 769-778.
10. Dadrasnia A., and Agamuthu P. Diesel fuel degradation from contaminated soil by Dracaena reflexa using organic waste supplementation. *International Journal of the Japan Petroleum Institute,* 2013b, 56 (4): 236-243.
11. EMD.Compound Specific Isotope Analysis 2011, http://www.itrcweb.org/documents/team_emd/CSIA_Fact_Sheet.pdf
12. Fleri, M. A. and Whetstone, G. T. In situ stabilisation/solidification: Project lifecycle. *Journal of Hazardous Materials,* 2007, *141*(2): 441-456.
13. FRTR. Cost and Performance Case Studies. FRTR Cost and Performance Reports. U.S. Army Environmental Center, Federal Remediation Technologies Roundtable.2012.
14. Gan S., Lau E.V., Ng H.K. Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). *Journal of Hazardous Materials*2009, 172 (2-3):532-549.
15. Gerhardt K.E., Huang X-D., Glick B. R., Greenberg B. M. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Science*, 2009, 176 (1):20-30.
16. Haderlein A., Legros R., Ramsay B. A. Pyrene mineralization capacity increases with compost maturity. *Biodegradation,* 2006, 17:293 – 302.
17. Ijah U. J. J., Safiyanu H., Abioye O.P. Comparative study of biodegradation of crude oil in soil amended with chicken droppings and NPK fertilizer. *ScienceWorld Journal,* 2008, 3 (2):63 – 67.
18. ITOPF. Oil tanker soill statistics. London: The International Tanker Owners Pollution Federation Ltd.,2011, pp. 1- 12.
19. Jain P.K., Gupta V.K., Gaur R.K., Lowry M., Jaroli D.P., Chauhan U.K., Bioremediation of Petroleum oil Contaminated Soil and Water. *Research Journal of Environmental Toxicology,* 2011, 5:1-26.
20. Khan, F. I., Husain, T. and Hejazi, R. An overview and analysis of site remediation technologies. *Journal of Environmental Management,* 2004, 71: 95–122.
21. Kim J., Min K.h., Cho K.S., Lee I. S., Enhanced Bioremediation and Modified Bacterial Community Structure by Barnyard Grass in Diesel-Contaminated Soil. *Environ Eng Res,*2007, 12:37- 45.
22. Kumar A., Bisht BS., Joshi V.vD., Dhewa T., Review on Bioremediation of Polluted Environment: A Management Tool. *International Journal of Environmental Sciences,* 2011,1(6):1079 - 1093.
23. Lee S-H., Oh B-I., Kim J-g., Effect of various amendments on heavy mineral oil bioremediation and soil microbial activity. *Bioresource Technology,* 2008, 99 (7):2578-258
24. López-Vizcaíno, R., Sáez, C., Cañizares, P. and Rodrigo, M. A. . The use of a combined process of surfactant-aided soil washing and coagulation for PAH contaminated soils treatment. *Separation and Purification Technology,*2012, *88*(0): 46-51.

25. Nie M., Wang Y., Yu J., Xiao M., Jiang L., Yang J., Fang C., Chen J., Li B., Understanding Plant-Microbe Interactions for Phytoremediation of Petroleum-Polluted Soil. *plos one,* 2011, 6(3):17961.
26. Okoh, A. I. Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. *Biotechnology and Molecular Biology Review,*2006, 1(2), 38-50.
27. Okoh, A. I. and Trejo-Hernandez, M. R.. Remediation of petroleum polluted systems: Exploiting the bioremediation strategies. *African Journal of Biotechnology,*2006, 5(25): 2520 – 2525.
28. Okoh, A. I.. Biodegradation of Bonny light crude oil in soil microcosm by some bacterial strains isolated from crude oil flow stations saver pits in Nigeria. *Afr. J. Biotech.,*2003, *2*(5): 104 - 108.
29. Perelo L. W., Review: In situ and bioremediation of organic pollutants in aquatic sediments. *Journal of Hazardous Materials,* 2010, 177 (1–3):81-89.
30. Qi, L., Ying Xu, C., ZhaoWei, W. and YuanPeng, W.. Study on the possibility of hydrogen peroxide pretreatment and plant system to remediate soil pollution. *Chemosphere,* 2004,57(10): 1439 - 1447.
31. Sayara T.A.S., Bioremediation of polycyclic aromatic hydrocarbons (PAHs)-contaminated soil: process evaluation through composting and anaerobic digestion approach. Universitat Autònoma de Barcelona. 2010.
32. Sharma S., Bioremediation: Features, Strategies and applications. *Asian Journal of Pharmacy and Life Science,* 2012, 2 (2):202 - 213.
33. Silva M.A.R., Mater L., Souza-Sierra M.M., Corrêa A.X.R, Sperb R., Radetski C.M., Small hazardous waste generators in developing countries: use of stabilization/solidification process as an economic tool for metal wastewater treatment and appropriate sludge disposal. *Journal of Hazardous Materials,* 2007, 147 (3): 986-990.
34. Sonawdekar S. (2012), Bioremediation: A boon to hydrocarbon degradation. International *Journal Of Environmental Sciences* 2 (4):2408 - 2423.
35. Wenzel, W. (2008), Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. *Plant and Soil* 321 (1):385-408.
36. Zahed, M. A., Aziz, H. A. and Isa, M. H. (2006). Oil Spill Cleanup Techniques in the Marine Environment. Paper presented at the International conference on Environmental Technology, Penang, Malaysia. Pp: 22-25.

Chapter 9

Pesticide residue evaluation in soil environment of mustard crop using chromatographic techniques

***S.P. Singh[1], Y.P. Singh[2], N.S. Bhogal[2] and Sandeep Kumar[1]**
[1]National Bureau of Plant Genetic Resources, New Delhi-110012
[2]Directorate of Rapeseed-Mustard Research (ICAR), Sewar, Bharatpur (Rajasthan)
***Corresponding author:** drspsingh64@gmail.com

Abstract

Insecticides i.e. imidacloprid 17.8 SL @ 7 ml/kg seed, imidacloprid 70 WS @ 7 g/kg seed, thiamethoxam 25WG @ 7 g/kg seed and fipronil 5 SC @ 7 ml/kg seed (all seed treatment), thiamethoxam 25WG @ 50 g a.i./ha and dimethoate 30 EC @ 300g a.i./ha (as foliar spray) were applied to determine the residue in the soil samples. The samples were collected (0-20 cm depth) from the treated and untreated plots after harvesting of the mustard crop in April during the year 2009-10. Analysis of imidacloprid and thiamethoxam was carried out with the help of HPLC (model SPD-M20A-LC20AT (Shimadju), Column C-18, 100A 5 µ 25 cm x 4.6 mm) while fipronil and dimethoate were analysed with Gas chromatograph. Before analysis, HPLC and GC were first standardized to separate the respective pesticides using standards.Overall methods used for extraction, clean up and estimation of residue were found to be satisfactory qualitatively and quantitatively. It was concluded from the study that residue level in the soil samples collected from the mustard field was below the detectable level (BDL). This study clearly indicates the safety of above insecticides to the soil microorganisms and other forms of soil biota.

Key words:

Insecticides, residuelevel evaluation, mustard seed, chromatography techniques

1. Introduction

Mustard is prominent oil producing crop being grown in India as well as in 52 other countries spreading over the six continents (Asia, Europe, Africa, Australia, North America and South America). More than three dozen of insects-pests are reported to infest the rapeseed-mustard crops at various phonological stages in India(Purwar and Sachan 2004).Lack of reasonable resistant source among the cultivated mustard, hazardous chemical pesticides are only practical tool to contain these dreaded insect-pests. Insecticides are used in mustard crop in both the ways, seed treatment at the sowing time and foliar spray at flowering and fruiting time.In the past many workers have evaluated a number of chemical insecticides against major insect-pests (Rohilla *et al.*, 2004; Sachan *et al.*, 2006; Singh *et al.*, 2007; Singh and Singh, 2009).

The use of chemical pesticides is very well known for environmental pollution, health hazards to human beings and residue in oil and cake (Singh, 2001; Kumar *et al*., 2001). In view of this, residue of some newly introduced molecules was evaluated in mustard soil to ascertain their safety index to soil microorganism and other form of soil biota.

2. Material and Methods

Field experiment was conducted at Directorate of Rapeseed-Mustard Research, Sewar, Bharatpur, Rajasthan, India during 2009-10 and analytical research work was carried out at Department of Entomology, Agricultural Research Stations at Durgapura, Jaipur inRajasthan.The insecticides used were imidacloprid 17.8 SL @ 7 ml/kg seed, imidacloprid 70 WS @ 7 g/kg seed, thiamethoxam 25WG @ 7 g/kg seed and fipronil 5 SC @ 7 ml/kg seed (all seed treatment), thiamethoxam 25WG @ 50 g a.i./ha and dimethoate 30 EC @ 300g a.i./ ha (as spray).Soil samples collected at a depth (0-20 cm) from the treated and untreated plots at harvest. Analysis of imidacloprid and thiamethoxam was carried out with the help of HPLC (model SPD-M20A-LC20AT (Shimadju), Column C-18, 100A 5 µ 25 cm x 4.6 mm). For imidacloprid analysis, HPLC operating parameters were as follows: mobile phase flow 1 ml/ min., ë=270, CAN: water (30:70), Rt.= 8.281 min., detector PDA, area std. 1ppm=94784 while for thiamethoxam HPLC operating parameters were: mobile phase flow 1 ml/min., ë=255, CAN: water (30:70), Rt.= 7.331 min. detector PDA, area std. 1ppm=106172. Fipronil and dimethoate were analysed with Gas chromatograph and operating parameters for fipronil were ECD detector, column (ECD)-DB-1, injector port temperature-280°C, detector temperature-290°C, column oven temperature programming-160°C-7°C/min-290°C-5 min. (hold), purge flow-3.0 ml/min., split ratio-5, Rt. (Fipronil)=9.454, area (1ppm std. fipronil)=2839040 while for dimethoate analysis NPD detector, column (NPD)-DB-5, injector port temperature-280°C, detector temperature-290°C, column oven temperature programming-160°C-7°C/min. 290°C-5 min. (hold), purge flow-3.0 ml/min., split ratio-5, Rt. (Dimethoate)=6.003, area (1ppm std. Dimethoate)=312010. The detection limit for HPLC was 0.02 ppm while for GC it was 0.05 ppm.

3. Results and Discussion

The terminal residue samples were analyzed and the result was listed in Table 1. Overall methods used for extraction, clean up and estimation of residue were found to be satisfactory qualitatively and quantitatively. The residue analysis of soil samples of treatments with pesticides *i.e.*imidacloprid 17.8 SL @ 7 ml/kg seed, imidacloprid 70 WS @ 7 g/kg seed, thiamethoxam 25WG @ 7 g/kg seed and fipronil 5 SC @ 7 ml/kg seed (all seed treatment),imidacloprid 17.8 SL @ 40 g a.i./ha, thiamethoxam 25WG @ 50 g a.i./ha, fipronil 5 SC @ 75 g a.i./ha and dimethoate 30 EC @ 300g a.i./ha (as spray) revealed that residue was found below detectable level in any of the treatment (Table 1). This shows the safety of these insecticides to the soil microorganisms and other forms of soil biota. Krishnaiah and Lal (1973) reported that dimethoate 30 EC @ 0.03% when used against mustard aphid left no residue in mustard seed and soil .Kumar *et al*., (2001) also observed that mustard seed treatment with imidacloprid (Gaucho 70 WS) @ 5 and 10 g a.i./ kg seed) and foliar spray of imidacloprid (Confidor 200 SL) @ 20 and 40g a.i./ha at 50% pod formation stage found no residues in

mustard seed and soil at harvest. Therefore imidacloprid treatment could be taken as safe for the crop protection. This study would be helpful in proper and safe application of these pesticides into mustard crop environment.

Table 1: Terminal residue of different insecticides in soil samples of treated mustard plots during 2009-10

Treatment	Dose	Average residue (ppm)
Imidacloprid 17.8 SL	7 ml/kg seed	BDL
Imidacloprid 70 WS	7 g/kg seed	BDL
Thiamethoxam 25 WG	7 g/kg seed	BDL
Fipronil 5 SC	7 ml/kg seed	BDL
Imidacloprid 17.8 SL	40 g a.i./ha	BDL
Thiamethoxam 25WG	50 g a.i./ha	BDL
Fipronil 5 SC	75 g a.i./ha	BDL
Dimethoate 30 EC	300 g a.i./ha	BDL
Water spray	-	BDL
Control	-	BDL

BDL = Below detectable level

4. Acknowledgements

The authors are highly thankful to the Director, Directorate of Rapeseed-Mustard Research, Sewar, Bharatpur (Rajasthan) for providing necessary facilities.

References

1. Krishnaiah, K. and Lal, R..Dissipation of dimethoate residues from sarson.*Himacal Journal of Agricultural Research*, 1973, **2**:24-28.
2. Kumar R., Dikshit, A K. and Prasad, S K..Bioefficacy and residues of imidacloprid in mustard.*Pesticide Research Journal,*2001, **13**(2): 213-217.
3. Purwar, J.P. and Sachan, G.C..Bio-efficacy of entomopathogenic fungi against mustard aphid, *Lipaphis erysimi* (Kalt.) on *Brassica campestris*.*Journal of Aphidology,*2004,**18**:5-10.
4. Rohilla, H.R., Bhatnagar, P. and Yadav, P.R..Chemical control of mustard aphid with newer and conventional insecticides.*Indian Journal of Entomology,*2004, **66**(1): 30-32.
5. Sachan, S.K., Chaudhary, A.S., Singh, D.V. and Singh, H..Efficacy and economics of some newer insecticides against mustard aphid, *Lipaphis erysimi* (Kalt.).*Indian Journal of Crop Science,*2006, **1**(1-2): 168-170.
6. Singh, P.K.. Control of mustard aphid, *Lipaphis erysimi* (Kalt.)(Homoptera: Aphididae) with minimum insecticide use.*Journal of Aphidology*,2001,**15**:139-142.
7. Singh, S.P. and Singh, Y.P.Bio-efficacy of pesticides against mustard aphid.*Annals of Plant Protection Sciences*, 2009, **17**(1): 240-242.
8. Singh, T.R., Singh, M.P., Singh, K.I., Devi, Th. B. and Singh, N.G.Comparative efficacy of certain neem products and conventional insecticides against *Lipaphis erysimi* (Kalt.) and their safety to its natural enemies in rapeseed.*Indian Journal of Entomology*,2007,**69**(3): 259-264.

Chapter 10

Study of the Genetic Mechanisms of Drug Resistance in *Mycobacterium tuberculosis* carried out in a Multispeciality Hospital in East Kolkata

***Indranil Roy, Ria Saha, Debasree Mazumder, Tapan Kumar Ghosh**
Department of Laboratory Services (Affiliated to College of American Pathology), Calcutta Medical Research Institute, Calcutta, India.
***Corresponding author:** dneel2004@rediffmail.com

Abstract

Multidrug-resistant tuberculosis (MDR-TB) is an increasing global problem, affecting the poor and the rich nations alike. The extent of the disease varies significantly from region to region. A prospective as well as retrospective study was conducted to determine the different mechanisms of drug resistance in *M. tuberculosis* detected in a variety of samples taken from patients who failed the primary treatment. 64 samples from the various indoor and outdoor patients who had a history of primary treatment failure were collected. Drug-sensitivity testing was performed by the PCR based Hain Test comprising of GenoType MTBDR*plus*and GenoType MTBDR*sl* for the first line drugs (rifampicin and isoniazid) and the second line drugs (aminoglycosides; kanamycin, amikacin, cyclic peptides; capreomycin and fluoroquinolones; ofloxacin, moxifloxacin)respectively and the molecular mechanisms underlying the drug resistivity was analyzed.

Keywords

Sensitivity, GenoType, Hain, MDR-TB, MTBDR*plus,* MTBDR*sl.*

1. Introduction

Tuberculosis is a global health problem. The emergence of Multidrug resistance (MDR-TB) and more, recently, of Extensive drug resistance are widely considered to be serious threats to global TB control (*MMWR Morb Mortal Wkly* Rep 2006,Migliori et. al 2007, Raviglione et. al 2007). MDR-TB is tuberculosis due to organisms which show resistance to both isoniazid and rifampicin, with or without resistance to other anti-TB drugs. As a result, this form of the disease is more difficult to treat than ordinary TB and requires up to 2 years of multidrug treatment. Mostly, it has been seen that the MDR cases arise due to physician error or patient non-compliance during treatment of susceptible TB or both (L. P. Ormerod, 2005). Studies conducted by S. K Sharma et.al in 2010 in India showed MDR- TB detected in 20.4% patients. The World Health Organization (WHO) fourth Global Project (2008)reported MDR-TB prevalence of 17.2 per cent among previously treated cases in India.

Resistance to isoniazid is due to mutations at one of the two main sites, in either the *katG* or *inhA* genes (conferring high level resistance &low level isoniazid resistance, respectively). Resistance to rifampicin is due to point mutations in the *rpo* gene in the beta subunit of DNA-dependent RNA polymerase. The **Geno**Type **MTBDR*plus*** assay simultaneously detects these specific mutations in the rpoB gene, katG gene as well as the inhA gene. When the first line drugs fail, second line drugs (quinolones, aminoglycosides, macrolides, capreomycin and others) are tried; the later being less efficacious, less convenient, more expensive and more toxic.

XDR-TB, on the other hand can be defined as MDR-TB in addition to resistance to any of the fluoroquinolones (such as ofloxacin or moxifloxacin) and to at least one of three injectable second-line drugs (amikacin, capreomycin or kanamycin). According to WHO, South Africa was burdened with 6.7% XDR for re-treated TB cases in 2011. Vijdea et.al in 2008 reported 5% patients with XDR in Denmark.

2. Materials &Methods

A retrospective as well as prospective study was conducted from November 2010 to August 2013 in the Department of Microbiology, Calcutta Medical Research Institute, Kolkata. A total of 64 samples (sputum, LRT samples, tissues, etc) from the various indoor and outdoor patients who had a history of primary treatment failure were taken. Of these samples, some of the Hain tests had been performed from smear positive cases and some from positive culture bottles which were on incubation in Bactec 460 and were indicated positive. Initially, the specific mycobacterial specie is identified using **Geno**Type® **Mycobacterium CM. Once** *M.tuberculosis* complex is detected in the sample, the sample is subjected to GenoType MTBDR*plus* and then, further to GenoType MTBDR*sl* for testing drug sensitivity towards the first line drugs (rifampicin and isoniazid) and the second line drugs (aminoglycosides; kanamycin, amikacin, cyclic peptides; capreomycin and fluoroquinolones; ofloxacin, moxifloxacin) respectively.Also, some of the samples suggested of Hain Test had also been cultured separately.

The HAIN test (GenoType MTBDR*plus* andGenoType MTBDR*plus*) provided by "the Hain Lifescience (GmbH, Germany)" is a reverse hybridization-based line probe assay (LiPA), which allows for the identification and detection of MTB along with rapid sensitivity testing for key first and second line anti-tubercular drugs endorsed by the WHO.

A similar Line Probe assay called the INNO-LiPA Rif.TB was introduced in the market by INNOGENETICS N.V.(Zwijndrecht, Belgium) in 2006. It had similar features and principle to the **Geno**Type **MTBDR*plus***, the only difference being the ability to detect the wild types and mutations in the *rpo*B gene region related to rifampicin resistance only. Detection of the isoniazid resistance was not included. **Geno**Type **MTBDR*plus*,** on the other hand accounts for the detection of both rifampicin and isoniazid resistance.

GenoType® **Mycobacterium CM,** GenoType MTBDR*plus*and GenoType MTBDR*sl*are based on the DNA Strip Technology. Some of the samples collected from the patients were tested for the presence of Acid Fast Bacilli by direct microscopy or they were sent for culture after decontaminating using the Petroff's method (using 4% NaOH solution). The smear

 ISBN: 978-81-928924-3-6

positive and the culture positive samples were further sent for the Hain test, which is basically a three step procedure.

DNA Extraction. Mycobacterial DNA is first extracted from the specimen. For this, the bacterial culture is first spun in a centrifuge (10000 x g).The pellet (100µl of the cell suspension) obtained is resuspended in 100-300 µl of water by vortexing. Incubation of this suspension or direct smear positive clinical samples is done at 95° C for 20 minutes followed by incubation in an ultrasonic bath. The sample is again spun down for around 5 minutes at full speed and finally, 5 µl of the supernatant is taken for the PCR (amplification step).

Amplification.A multiplex amplification step is carried out where the extracted DNA is amplified with the help of biotinylated primers via Polymerase Chain Reaction in a thermal cycler.

Per tube amplification mix contains 35 µl PNM (pink), 5 µl of 10X Polymerase Incubation Buffer, 2 µl of 25mM $MgCl_2$, 2 unit thermostable DNA polymerase, 3 µl molecular grade water to obtain volume of 45 µl (not considering volume of enzyme). Next, 5 µl DNA solution leading to a final volume of 50 µl is added. The tubes are now loaded into the thermal cycler) and it is programmed. Amplification occurs in cycles via PCR.

	Culture sample	**Clinical sample**
15 minutes; 95° C	1 cycle	1 cycle (hold)
30 seconds; 95° C 02 minutes; 58° C	10 cycle	10 cycle (cycle 1)
25 seconds; 95° C 40 seconds; 53° C 40 seconds; 70° C	20 cycles	30 cycles (cycle 2)
08 minutes; 70° C	1 cycle	1 cycle (hold)

Reverse Hybridization. Theamplified biotinylated DNA material is hybridized with specificoligonucleotide probes immobilized as parallel lines on membrane based strips.Streptavidin labeled with alkaline phosphatase is added which binds to the hybrid previously formed. Incubation with an appropriate chromogen results in a purple/brown precipitate, which can be visually interpreted.

The Twincubator is prewarmed to 45° C. HYB and STR solutions are prewarmed to 37-45° C before use.The remaining reagents are also warmed to room temperature, except for the CON-C/ SUB C (kept at room temperature). Also, using a suitable tube, dilute the conjugate concentrate and substrate concentrate 1:100 with respective buffer (CON-C with CON-D, SUB-C with SUB-D) in amounts needed. These are now mixed well and brought to room temperature.20 µl of DEN solution (blue) is dispensed in a corner of each of the well. To the solution is added 20 µl of Amplification sample and is mixed well by pippetting up and down. Incubation is done at room temperature for 5 minutes. Next, to each well, 1 ml of the prewarmed HYB buffer (green) is added. The strip is

placed in each well and the trays then put in the shaking water bath (300 rpm) at 45° C for 30 minutes. The HYB buffer is then completely aspirated.1 ml of STR wash solution (red) is added to each strip and incubated for 15 minutes at 45° C in shaking water bath. STR solution is aspirated. The strips are washed once with 1 ml of Rinse Solution (RIN) for 1 minute on shaky platform. Next is added, 1 ml of dilute Conjugate to each strip. Incubation is done at 25° C for 30 minutes. The solution is removed and washed (twice with RIN for 1 minute and then once with 1 ml of molecular grade water on shaking platform). One ml of dilute Substrate is added to each strip and incubated while protected from light without shaking for 5 minutes. The reaction is stopped by rinsing twice with distilled water. Using tweezers, the strips are removed from the tray and dried between two layers of absorbent paper.

3. Interpretation of Results

3.1 GenoTypeMycobacterium CM:

Each strip consists of 17 reaction zones (bands), including three controls (conjugate, universal and genus controls), and fourteen probes for fourteen different NTM species. Results were interpreted according to the pattern of the bands formed.

3.2 GenoTypeMTBDR*plus*:

Each strip consists of 27 reaction zones (bands), including six controls (conjugate, amplification, M. tuberculosis complex, rpoB, katG, and inhA controls), eight rpoB wild-type (WT) and four mutant (MUT) probes, one katG wild-type and two mutant probes, and two inhA wild-type and four mutant probes (Figure). Results were interpreted according to the pattern of the bands formed.

3.3 GenoTypeMTBDR*sl:*

Each strip consists of 22 reaction zones (bands), including six controls (conjugate, amplification, M. tuberculosis complex, gyrA, rrs and embB controls), three gyrA wild-type (WT) and six mutant (MUT) probes, two rrs wild-type and two mutant probes, and one embB wild-type and two mutant probes. Results were interpreted according to the pattern of the bands formed.

3.4 Results

64 isolates included in the present study were analysed by **GENOTYPE MTBDR*plus*for the determination of their drug susceptibility pattern. Out of the 64 isolates analysed,**9% were resistant to INH, 2% were resistant.to RIF, 31% were the MDR-TB cases and 58% were susceptible to both INH and RIF. Furthermore, only 1 out of 6 of the INH monoresistant isolates showed low level resistance towards the drug, the rest of the 5 showing high level INH resistance. Two cases were resistant to INH, rifampicin, aminoglycosides (rrs mutation) and fluoroquinolones (gyrA mutation) suggesting the possibility of XDR. On further analysis by GenoType MTBDR*sl,* 28 % of the isolates turned out to be resistant to ethambutol with 26% being resistant to fluoroquinolones and 10% were resistant to the aminoglycosides. Among the MDR cases (20%), GenoType MTBDR*sl* detected 53% isolates as EMB resistant, 47% as FLQ resistant and 12% AG resistant (figures 1, 2 and 3).

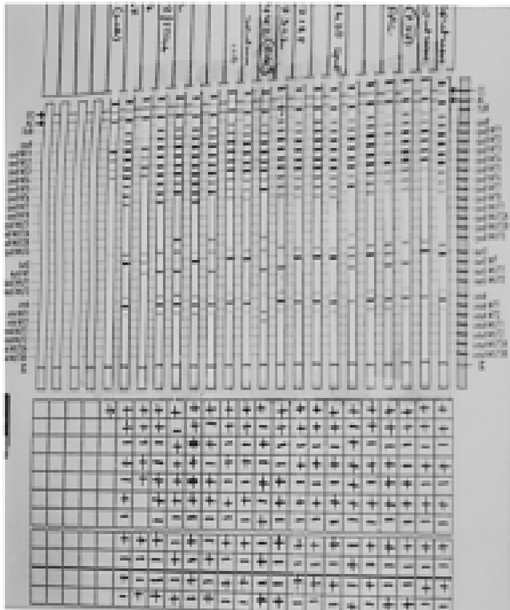


Fig.1: GENOTYPE MTBDR*plus* STRIP

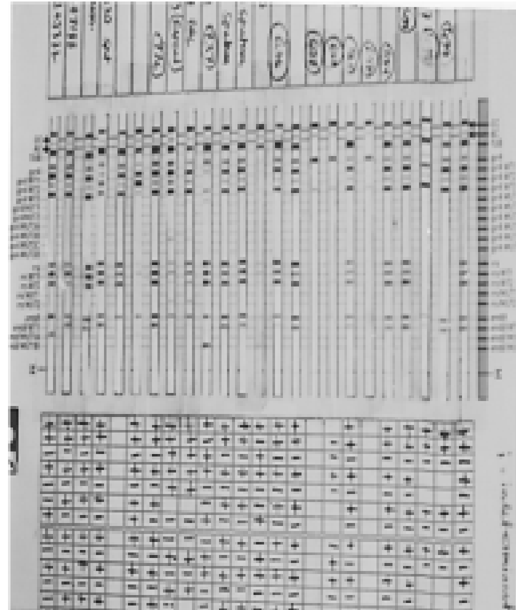


Fig.2: GENOTYPEMTBDR*sl* STRIP

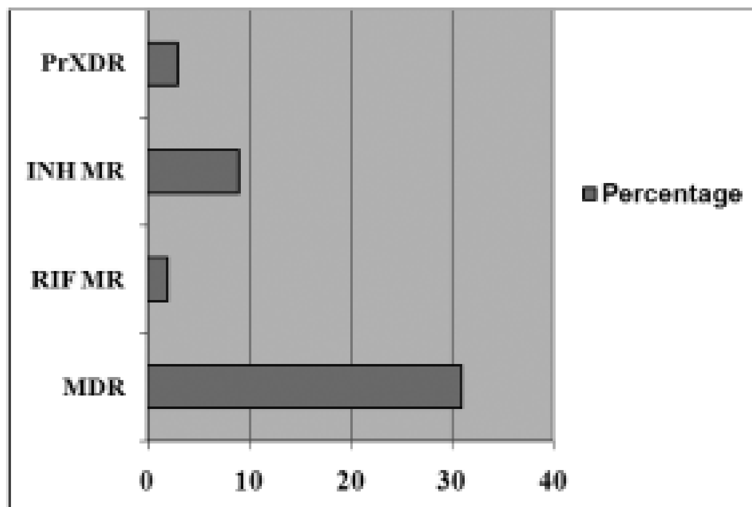


Fig. 3

The different mechanisms of resistance to different drugs in *Mycobacterium tuberculosis* strains is attributed to the different patterns of gene mutations they show. Mutations in the different gene regions in the *M. tuberculosis* strains result into their resistance towards the various drugs. For example- mutation in D516V, H526Y, H526D or S531L gene regions results in resistance of the *M. tuberculosis* strain towards the drug rifampicin. These mutations are visible in Genotype MTBDR*plus* in theform of bands - *rpoB* MUT1, *rpoB* MUT2A, *rpoB* MUT2B and *rpoB* MUT3 respectively (table 1).

3.5 Observation (refer to table 1):

- **In MDR cases (n=20):**

  frequency of the S531L mutation in *rpoB* - 19 [95%] of 20 (highest)
  frequency of the S315T1 mutation in *katG*- 17 [85%] of 20 (second highest)
  frequency of the H526Y mutation in *rpoB*- 01 [5%] of 20
  frequency of the H526D mutation in *rpoB*- 01 [5%] of 20
  frequency of the S315T2 mutation in *katG*- 01 [5%] of 20
  frequency of the T8C mutation in *inhA*- 01 [5%] of 20
  frequency of the C15T mutation in *inhA*- 03 [15%] of 20

- In INH MR cases (n= 06)
  (high level resistance):
  frequency of the S315T1 mutation in *katG*- 05 (83%) of 06
  (low level resistance):
  frequency of the T8C mutation in *inhA*- 01 (17%) of 6
  frequency of the T8A mutation in *inhA*- 01 (17%) of 6
  In RIF MR cases (n= 01)
  WT8 band was missing suggesting alteration at gene region 530-533.

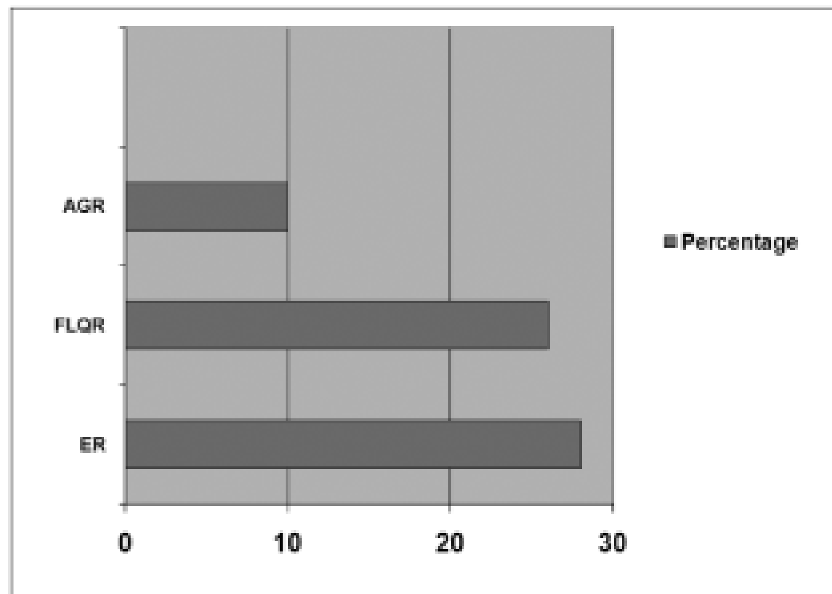


Fig. 4: Respective drug resistance observed during susceptibility testing by GenoType MTBDR*sl*

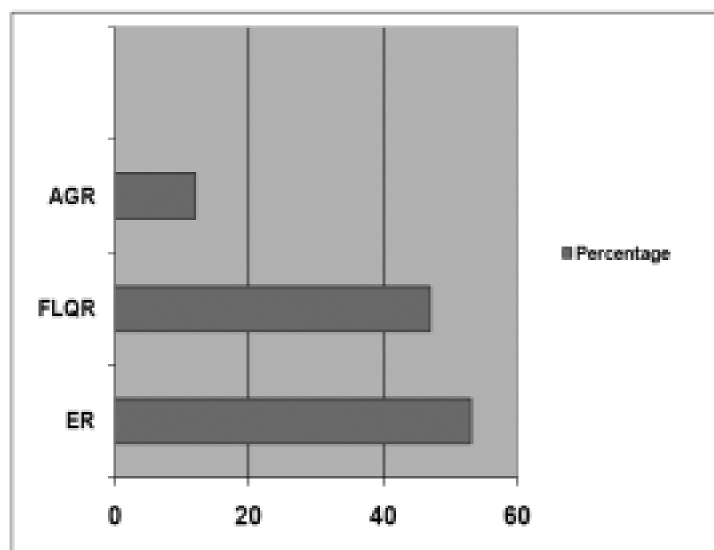


Fig. 5: Percentage of ethambutol resistance, fluoroquinolone resistance and aminoglycoside resistance in MDR cases.

Table 1: Pattern of Gene Mutationsin Resistant*Mycobacterium tuberculosis* Strains Using Genotype MTBDR*plus* ASSAY[n=64] [Values are numbers, with percentages in parenthesis].

Gene	Band	Gene Region or Mutation	MDR (n=20)	INH Monoresistant (n=6)	RIF Monoresistant (n= 1)
rpoB	WT1	506–509	19 (95)	06 (100)	1 (100)
	WT2	510–513	19 (95)	06(100)	1 (100)
	WT3	513–517	19 (95)	06(100)	1 (100)
	WT4	516–519	20 (100)	06(100)	1 (100)
	WT5	518–522	20 (100)	06(100)	1 (100)
	WT6	521–525	19 (95)	06(100)	1 (100)
	WT7	526–529	19 (95)	06(100)	1 (100)
	WT8	530–533	0 (0)	06(100)	0 (0)
	MUT1	D516V	0 (0)	0 (0)	0 (0)
	MUT2A	H526Y	01(5)	0 (0)	0 (0)
	MUT2B	H526D	01 (5)	0 (0)	0 (0)
	MUT3	S531L	19 (95)	0 (0)	0 (0)
katG	WT	315	02(10)	03 (50)	1 (100)
	MUT1	S315T1	17 (85)	05 (83)	0 (0)
	MUT2	S315T2	01(5)	0(0)	0 (0)

inhA	WT1	–15/–16	17 (85)	06(100)	0 (0)
	WT2	Š8	18 (90)	05 (83)	1 (100)
	MUT1	C15T	03 (15)	0 (0)	0 (0)
	MUT2	A16G	0 (0)	0 (0)	0 (0)
	MUT3A	T8C	01 (5)	01 (17)	0 (0)
	MUT3B	T8A	0 (0)	01 (17)	0 (0)

4. Discussion

When MDR-TB is detected, the next step towards the treatment of the patient is to determine the susceptibility of the strain to the remaining first-line drugs, i.e., streptomycin, ethambutol, and pyrazinamide, and to second-line drugs; aminoglycosides/ cyclic peptides (kanamycin, amikacin, and capreomycin) and fluoroquinolones (ofloxacin, moxifloxacin). In the present study, this has been done by the performance of the Hain Test which assesses the susceptibility of the strain to both first-line and second-line drugs.**Out of the 64 isolates analysed** by **GENOTYPE MTBDR*plus*,** MDR-TB was detected in 31% of the patients. The present study has also detected 2 (3%) patients with probable XDR.

As we know by now that the different mechanisms of resistance to different drugs in *Mycobacterium tuberculosis* strains is attributed to the different patterns of gene mutations they show, a study has been performed on the pattern of the gene mutations in resistant *Mycobacterium tuberculosis***using Genotype MTBDR*plus*assay.** During this study**, it was seen that in the MDR cases,** the frequency of the S531L mutation in *rpoB* was the highest (95%). This particular mutation has been observed as the most prevalent mutation in the *rpoB* gene in many other studies (Barnard et. al, 2002 and Tracevska et.al, 2002). The frequency of the S315T1 mutation in *katG* on other handwas (85%) the second highest. Other mutations observed include the C15T mutation in the *inhA*, the frequency being 3 [15%] of 20 (third highest mutation) and mutations in the H526Y and H526D in *rpoB* (both 5%). Also, there has been mutation in the S315T2 in *katG and* T8C in *inhA* (both 5%). An interesting fact noted during the study was that WT8 band, coding for the gene region 530–533 in *rpoB* was missing in all the 20 isolates. Also, no mutation has been observed in the gene regions D516V and H526Y. Similar was the case with A16G and T8A in *inhA* which showed no mutation. Eight isolates out of 20 had the *katG* WT gene missing which is significant. Only 1 out of 64 isolates tested for susceptibility in the present study had turned out to be Rifampicin mono resistant. This particular isolate was found to have the *rpoB*WT8 band missing which accounts for the gene region 530–533. On the other hand 6 out of 64 isolates were found to be isoniazid mono resistant with only 1 isolate showing low level resistance towards isoniazid, while the rest of the 5 being high level isoniazid resistant cases. 3 of the isolates had *katG* WT gene missing and all had *inhA* WT1 present in them. Most of the mutations seen or to be precise, all the high level isoniazid resistant isolates had mutations in S315T1 present in *katG* whereas the 1 low level INH resistant isolate had mutation in regions T8C and T8A present in *inhA*. Over all, the present study employing rapid, simple, MTBR*plus* assay helps in gaining a better understanding of MDR-TB. Both MTBR*plus* assay and detection of XDR*M.tuberculosis*

can be of a substantial progress regarding the diagnosis of drug resistance to strengthen the management of patient therapy and prevention of transmission (Hillemann et. al, 2009). Multi drug resistance serves as an epidemiologic marker for the identification of clusters of the resource constrained countries until the preventive measures brought to bear. The future goal is deeply involved in providing a better insight to the public regime, spreading awareness of biosafety, observing the frequency of drug intake in stupor destitute and endemic areas, preventing predisposition to multidrug resistance. On the other hand, accelarating our research, producing investigational, adavanced form of new TB drugs to use these drugs in novel combinational regimen facilating health while preventing multidrug resistance.

Abbreviations:

AGR = aminoglycoside resistance

ER = ethambutol resistance

FLQR = fluoroquinolone resistance;

INH = isoniazid

INH MR = isoniazid monoresistance

MDR = multidrug resistance

PrXDR = Probable extensive drug resistance

RIF = rifampicin

RIF MR = rifampicin monoresistance

References:

1. Centers of disease control and prevention. Emergence of *Mycobacterium tuberculosis* with extensive resistance to second line drugs worldwide, 2000-2004. *MMWR Morb Mortal Wkly* Rep 2006; 55: 301-305
2. Migliori GB, Loddenkemper R, Blasi F, Raviglione MC. 125 years after Robert Koch discovery of the tubercle bacillus: the new XDR threat. Is “science” enough to tackle the epidemic? *Eur Respir* J 2007; 29: 423-427
3. Raviglione MC, Smith IM. XDR tuberculosis-implications for global pulic health. *N Engl J Med* 2007; 356: 656-659
4. L. P. Ormerod. Multidrug-resistant tuberculosis (MDR-TB): epidemiology, prevention and treatment, 2005, 73-74.
5. Surendra K. Sharma, Sanjeev Kumar, P.K. Saha, Ninoo George, S.K. Arora, Deepak Gupta, Urvashi Singh, M. Hanif & R.P. Vashisht. Prevalence of multidrug-resistant tuberculosis among Category II pulmonary tuberculosis patients,2011, pp 312-315.April 5, 2010.
6. World Health Organization. Anti-tuberculosis drug resistancein the world; Report no. 4; Geneva, Switzerland. WHO/HTM/TB/2008.394. Available from: http://whqlibdoc.who.int/hq/2008/WHO_HTM_TB_2008.394_eng.pdf , accessed on
7. Marinus Barnard, Heidi Albert, Gerrit Coetzee, Richard O’Brien and Marlein O. Bosman. Rapid Molecular Screening for Multidrug-Resistant Tuberculosis in a High-Volume Public Health Laboratory in South Africa, American Journal ofRespiratory and Critical Care Medicine.2008.
8. Mark Daku. Tuberculosis and drug resistant Tuberculosis inSouth Africa, AFRICA PORTAL ,2011, www.africaPorTal.org

9. R. Vijdea, M. Stegger, A. Sosnovskaja, Å. B. Andersen, V. Ø. Thomsen, and D. Bang. Multidrug-resistant tuberculosis: rapid detection of resistance to rifampin and high or low levels of isoniazid in clinical specimens and isolates, Eur J Clin Microbiol Infect Dis,2008, DOI 10.1007/s10096-008-0548-9
10. T. Tracevska, I. Jansone, L. Broka, O. Marga, and V. Baumanis. Mutations in the *rpoB* and *katG* Genes Leading to Drug Resistance in *Mycobacterium tuberculosis* in Latvia, 2002, 40(10): 3789–3792.
11. Hillemann D, Rusch-Gerdes S, Richter E, Feasibility of GenoType MTBR*sl* assay for Fluoroquinolone, Amikacin-Capreomycin and Ethambutol Resistance Testing of *MycobacteriumTuberculosis* Strains and Clinical Specimens, 2009; 47: 1767-1772

Chapter 11

Remediation of Kolkata Sewage through Waste Water Fishery and Open Circulation: A case Study

***Jyotiskona Barik, Debabrata Mukherjee, Mrinmoy De, Shreyasee Roy, Gourab Choudhury**
Institute of Ecotoxicology and Environmental Science, Kolkata, India
***Corresponding author:** bjyotiskona@gmail.com

Abstract

Kolkata is a congested and old metropolitan city of India, having 45 lakhs population within 185 km^2 area. Wastewater flows through underground sewers to pumping stations located in eastern fringe of the city, and is then pumped into open channels. Thereafter, the sewage and wastewater is drawn into the fisheries of the East Kolkata Wetland (EKW). In EKW, organic compounds of the sewage and wastewater are biodegraded. A network of channels is used to supply untreated sewage and to drain out the effluent. Present study deals with water conditions within the city, in EKW, effluent from EKW and at the end of the sewerage canal where it connects with riverine systems. The most important function performed by these wetlands is to reduce the Biological Oxygen Demand (BOD) from the daily 1,100 million liters per day (ML/d) of wastewater discharged from the city. BOD concentration within city at Topsia is about 150mg/L whereas in EKW pond water, after three days of waste water feeding is 90 to 110 mg/L. Bantala (Effluent from EKW), shows BOD within the range of 30 to 56mg/L. Sample from Ghusighata lock gate gives BOD below 20mg/L. The Dissolved Oxygen (DO) concentrations from upstream to downstream are 1.7 to 2.1 mg/L at city, 2 to 3.4 mg/L at EKW , 4.5 to 5 mg/L at Bantala and 6 mg/L at the connection point of sewerage canal and riverine system. This result shows the improvement of water quality through waste water fishery pond and flows of water through a long route. This is a low technology treatment but very effective in a natural way.But this very rare natural "wetland of international importance" is under immense threat because of urbanization and encroachment. Immediate implementation of effective policies is required in order to save this system.

Keywords

Kolkata, Sewage system, East Kolkata Wetland, BOD, DO

1. Introduction:

Kolkata, formally known as Calcutta, located on the east bank of Hooghly River, is the capital of Indian state of West Bengal and serves as principal commercial, cultural and

educational centre of eastern India. It is a very old city which started its formation in late 17th century by British. This city served as capital of India until 1911 and following Indian independence in 1947 it witnessed several decades of relative stagnation resulting in lack of environment cautious development approach. As of 2011 the city's population was 4.5 million; including urban clusters, which comprises the city and its suburbs it was 14.1 million making it third most populous metropolitan area in India. This puts increased pressure on old sewage system of this metropolitan as this huge population consumes huge resources and produces huge amount of sewage as well.

In spite of producing this huge amount of sewage, more than 125 years of old Kolkata's basic sewage structure can process this because this sewage process includes open channels, East Kolkata Wetlands (EKW) and sewage canals which meet with riverine systems. A wetland is "an ecosystem that arises when inundation by water produces soils dominated by anaerobic processes, which, in turn, forces the biota, particularly rooted plants, to adapt to flooding" (Keddy, 2010). Wetlands are usually the transitional zones between aquatic and non-aquatic ecosystems. These zones are seasonally or permanently saturated with salt, fresh or brackish water (Gupta, 2013).EKW is situated in the eastern fringes of Kolkata city (22^{0}29' to 22^{0} 34'1.45"N and 88^{0} 25'2.33" to 88^{0}28'1.05"). EKW was designated as "wetland of international importance" (listed by the Ramsar Bureau In August 2002). It is an excellent combination of environmental protection and development where a unique and complex ecological process has been adopted by mastering the resource recovery activities (WWF 2006).It has been delivering environmental benefits worth $38.54 million (Bhattacharyya et al. 2008). The most important function performed by EKW is to reduce the Biological Oxygen Demand (BOD) of sewage water which flows into EKW through open channels after sewages are pumped into those channels. Thus EKW works as works as natural sewage processing system.

EKW also supports livelihood of approximately15 million inhabitants directly with gender ratio of about 919:1000 (Bhattacharyya et al. 2008). This natural wetland supports the world's largest wastewater fish culture covering an area of 4000 Ha and contributes a significant percentage of the total Indian economy (Mukherjee, 2011). Nearly 10,915 tons of fish is produced annually in about 286 wastewater fed fishponds (Mukherjee, 2011). In addition, the farmlands of EKW produce rice and vegetables which helps in meeting the demands of metropolitan populations.

Three major canals namely Bagjola Khal, Kestopur Khal and Dry weather flow canal cross the heart of the city diagonally and almost touch the East Kolkata Wetland, acting as feeding canal for EKW waste water fisheries. Apart from those big sewage canals there are several small sewages which are connected with the major canals and flow through them. These sewage canals were connected with Hooghly River in the west and Kulti gang in the east as per their originality. But in present scenario, these canals are blocked in different places and no direct connection can be seen with River Hooghly.

 ISBN: 978-81-928924-3-6

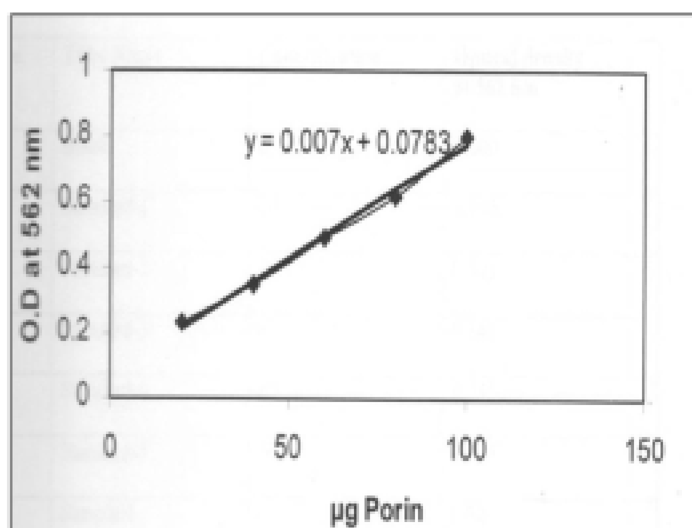


Fig 1: East Kolkata Wetland

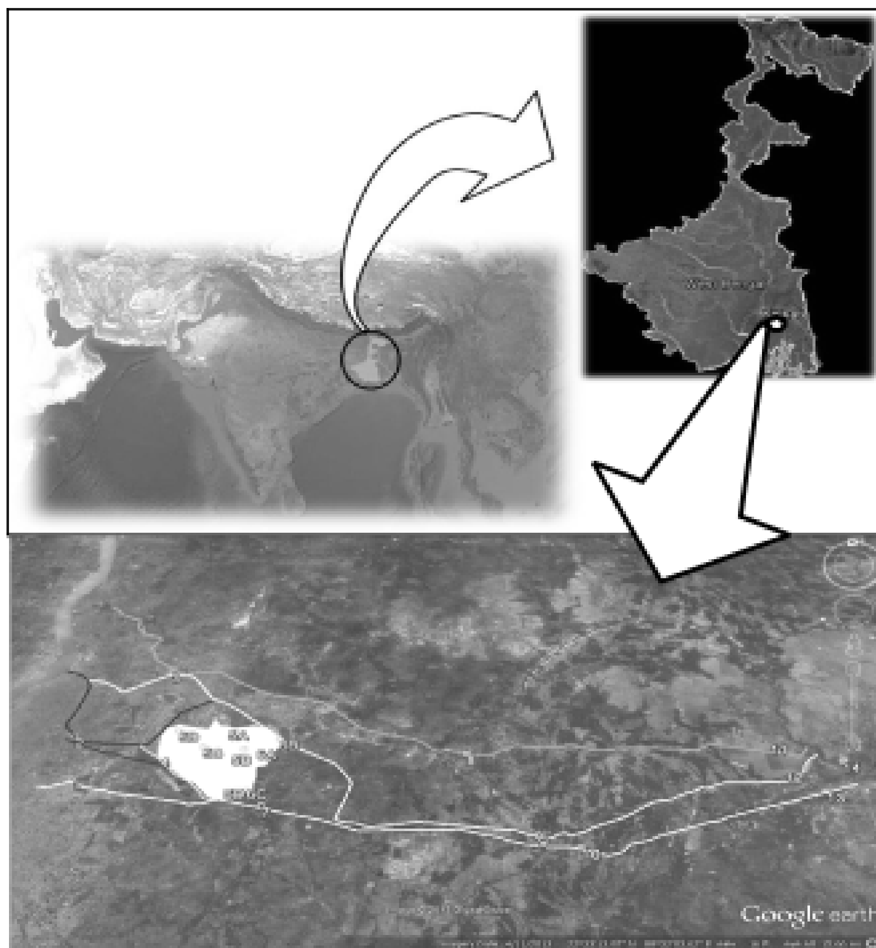
This study deals with characterization of water quality in sewage canals within the city as well as the water quality of those canals when they connect with the estuarine system very nearer to Sundarbans. No big investment is there to treat the sewage water from a big metropolitan city like Kolkata.

2. Study Site:

20 different locations on major sewage canals had been selected for sample collection.Details of those positions are mentioned in below chart.

<table>
<tr><th>Sampling Point</th><th>Latitude</th><th>Longitude</th><th colspan="2">Position</th></tr>
<tr><td>1</td><td>22°32’20.44"N</td><td>88°23’16.59"E</td><td>Present on Dry Weather Flow mainly carrying the effluent from Topsia tannery industrial belt.</td><td rowspan="3">Raw Sewage</td></tr>
<tr><td>2</td><td>22°33’47.30"N</td><td>88°22’58.46"E</td><td>Present on Beliaghata canal which ultimately meets with Kestopur Khal.</td></tr>
<tr><td>3</td><td>22°36’12.05"N</td><td>88°24’52.33"E</td><td>Present on the junction point of Kestopur Bagjola Khal.</td></tr>
<tr><td>4</td><td>22°32’42.85"N</td><td>88°25’23.93"E</td><td>Location just before the EKW entry point.</td><td rowspan="3">EKW initial</td></tr>
<tr><td>5A</td><td>22°33’34.82"N</td><td>88°27’00.55"E</td><td>Inlet point of EKW Bhery.</td></tr>
<tr><td>5B</td><td>22°33’08.29"N</td><td>88°27’08.75"E</td><td>Inlet point of EKW Bhery.</td></tr>
</table>

5C	22°33’15.67"N	88°26’08.74"E	Inlet point of EKW Bhery.	
5D	22°33’52.20"N	8°25’28.05"E	Inlet point of EKW Bhery.	
6A	22°32’52.15"N	88°27’45.83"E	Outlet point of EKW waste water fisheries pond.	EKW Final
6B	22°33’27.56"N	88°27’57.31"E	Outlet points of EKW waste water fisheries pond.	
6C	22°31’45.35"N	88°27’21.89"E	Outlet point of EKW waste water fisheries pond.	
6D	22°31’37.04"N	88°27’11.02"E	Outlet point of EKW waste water fisheries pond.	
7	22°31’17.50"N	88°27’44.34"E	Point where effluent from EKW is again released in canal water.	
8	22°32’43.89"N	88°32’27.55"E	Present on Bagjola Kestopur DWF canal.	Mid way to Discharge
9	22°30’07.62"N	88°33’49.36"E	Present on Bagjola Kestopur DWF canal.	
10	22°29’44.90"N	88°34’41.08"E	Present on Bagjola Kestopur DWF canal.	
11	22°32’27.59"N	88°40’00.33"E	Discharge point of 8	Final Discharge
12	22°31’31.47"N	88°40’01.84"E	Discharge point of 9	
13	22°31’07.58"N	88°40’33.53"E	Discharge point of 10	
14	22°32’03.90"N	88°41’18.78"E	Point from river named as Kulti Gang where all the sewage canals are discharged.	



Map 1: Location Map of the Study Area ShowingDifferent Sewage Canal and Sampling points on them

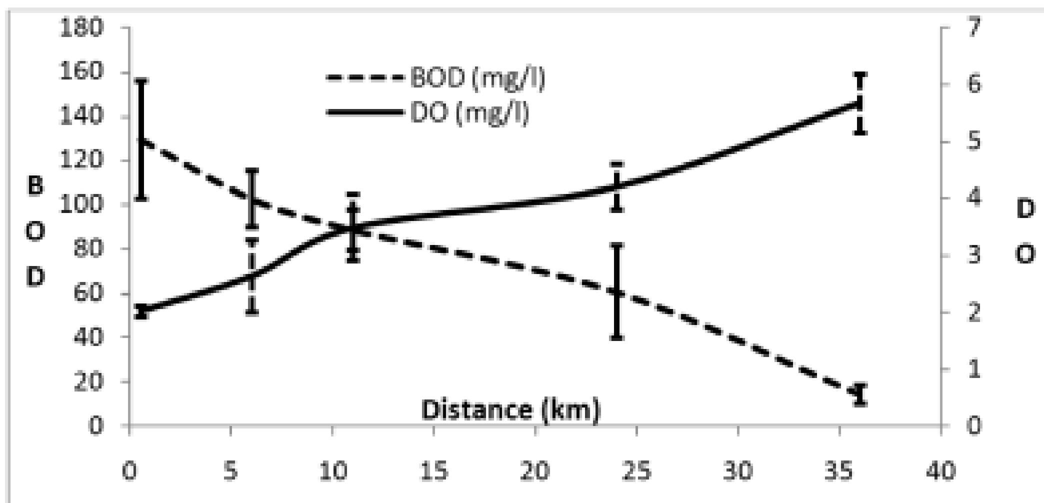
3. Materials and Methods:

GPS had been used for selecting the locations according to their position within the city and out of the city. Chemicals from Merck had been used to carry out the whole procedure.The water samples were collected in a 250ml Borosil DO bottles from different sampling sites as discussed above. 2ml 48% $MnSO_4$ and 2 ml alkaline KI were added to fix the samples for estimating BOD and DO. Five days dilution BOD test method (APHA, 1995) had been used to estimate BOD. DO was measured through Winkler's Iodometric method.

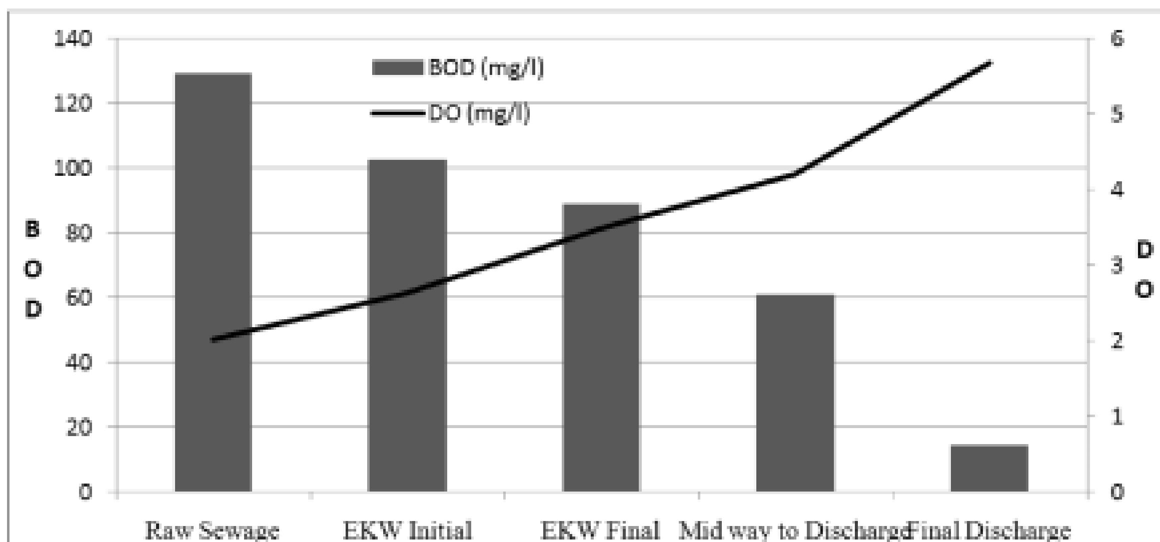
4. Results and Discussions:

Tropical regions are known for treating sewage in ponds. Flat bottom shallow waste water fed fish ponds are known as Bhery which are mostly seen in the eastern part of India. Bhery is very effective for integrated resource recovery. Waste water is naturally processed in Bhery. Then that processed water is utilized for cultivation of fishes. Then that water and the fertile effluent are used for agriculture in nearby fields(Pradhan et al. 2008). Water samples from sewage canal within the city shows the worst quality. BOD concentration within city at Topsia

is about 150mg/L whereas in EKW pond water, after three days of waste water feeding is 90 to 110 mg/L. Bantala (Effluent from EKW), shows BOD within the range of 30 to 56mg/L. Sample from Ghusighata lock gate gives BOD below 20mg/L. The Dissolved Oxygen (DO) concentrations from upstream to downstream are 1.7 to 2.1 mg/L at city, 2 to 3.4 mg/L at EKW , 4.5 to 5 mg/L at Bantala and 6 mg/L at the connection point of sewerage canal and riverine system. The water improvement rate within EKW fisheries pond is quite higher as those pond helps to settle down the organic load and increase the productivity of that water body. When water contains a good phytoplankton population, automatically DO level increases and water quality became good.



Graph 1: BOD-DO vs. Distance



Graph 2: BOD-DO in Different Locations

5. Conclusions

EKW is rightly designated as "wetland of international importance". There is probably no other instance of such a natural waste recycling system anywhere in the world. It works as natural sewage processing system as well it gives economic means to inhabitant. But for the past few years EKW is under immense pressure because of urbanization results in massive encroachment and rampant construction. If this situation continues, it will gradually destroy the natural sewage system and cause a threat of containing variety of chemicals and heavy metals violating existing environmental laws. It will also be a threat for health hazard through food chain. In such scenario, huge amount of money will have to be spent to build and run sewage treatment plants which this city will find difficult given the socio-economic scenario of this city.To avoid this, effective conservation and management policies need to be implemented by administrative authorities and other various stakeholders keeping in mind biodiversity as well as livelihood of local people. Then it can surely be the largest natural ecosystem in the whole globe which can convert sewage to wealth.

References:

1. Asian Development Bank. Human Resource Policy and Economic Development. Manila, Asian Development Bank.1991.
2. Bhattacharyya A., S Sen, P. K. Roy, A. Majumdar.. Edited by Sengupta M, Dalwani R. 2008. A Critical Study on Status of East Kolkata Wetlands with Special Emphasis on Water Birds as Bio-Indicator. Proceedings of Taal2007: The 12th World Lake Conference: 2007, 1561-1570.
3. EIP Working Report 5.. Sustaining Calcutta: Present Status Report of the Urban Peoples Environment (Calcutta, CMW & SA)., 1996.
4. Ghosh Dhrubajyoti& Christine Furedy. Resource Conserving Traditions and Waste Disposal: The Garbage Farms and Sewage-fed Fisheries of Calcutta. *Conservation and Recycling*, 1984, Vol. 7, No. 2-4.
5. Gupta S.Changing Land-use of East Kolkata Wetland, India. *International Journal of Applied Research & Studies,* 2013, Vol. II, Issue 3, ISSN 2278 – 9480. 352p.
6. Keddy P. A..*Wetland Ecology: Principles and Conservation*. Cambridge University Press, Cambridge.2010.
7. Mukherjee D. P.. Stress of urban pollution on largest natural wetland ecosystem in East Kolkata-causes, consequences and improvement. *Archives of Applied Science Research, ISSN 0975-508X,*2011, 3 (6):443-461.
8. Pradhan A, P. Bhaumik, S. Das, M. Mishra, S. Khanam, B. A. Hoque, I. Mukherjee, A. R. Thakur, S. R. Chaudhuri. Phytoplankton Diversity as Indicator of Water Quality for Fish Cultivation, *American Journal of Environmental Sciences,* 2008, *4 (4):* 406-411p.
9. WWF. East Kolkata Wetland Base line document, May23.2006.

Chapter 12

Loss of biodiversity due to Impact of Prawn Seed Collection - a crucial Practice of Fisheries Industry in Indian Sundarbans

***Mayukh Barik[1], Shreyasee Roy[2], Mrinmoy De[3], Gourab Choudhury[4], Jyotiskona Barik[4] and Debabrata Mukherjee[4]**

[1]Department of Environmental Science, Vidyasagar University, West Bengal, India

[2]Department of Botany, Vidyasagar University, West Bengal, India

[3]Ranaghat Bharati High School, Kamarpara, Ranaghat, West Bengal, India

[4]Institute of Ecotoxicology and Environmental Science, Kolkata, India

***Corresponding author:** mayukh.barik@gmail.com

Abstract

Sundarban is a unique place for biodiversity in India and also in the world. It has reclaimed habitat of 5366 km^2 of the total area of 9630 km^2. Local inhabitants of this area are vastly dependent on the natural resources of this zone for livelihood. One of the important ways to generate their livelihood is the fishing. Though the fresh water or the estuarine fishing is major livelihood, prawn seed collection is also an important one. But fishing industry is getting much trouble because of unplanned and unorganized fishing of the fishermen and some unscientific ways of seed collection. In case of prawn seed collection, mainly the target is to collect the prawn seedsof about PL-20 (Post Larval stage - 20 days) size for immediate cash generation, as their market value is quite good. Moreoverthis is now receivinggood interest from the fish farmers for its export potentialities. But during catching process of prawn seeds, some ecologically and economically significant faunal species of very early stages of their growth, are wasted as they are not released back to the water, causing a huge biodiversity loss.This study reveals the loss of biodiversity due to offhand prawn seed collection in some important sites of Indian Sundarbans.Proper knowledge transfers and awareness about seed collection, importance of biodiversity and the fish culture practice can protect the environment from this loss.

Keywords:

Sundarbans, Prawn seed collection, Biodiversity loss.

1. Introduction:

TheIndian Sundarbans is within the latitude (21^{0}31$^/$to 22^{0}30$^/$) N and longitude(88^{0}10$^/$ to 89^{0}51$^/$)E, in the state of West Bengal of India including the districts of North and South 24 Parganas. The region is limited by Raimangal River bordering Bangladesh in the east, the Hooghly River in the west,"Dampier-Hodges line" in the north and the Bay of Bengal in the

south.Now a day, Sundarbans has been declared asSundarban Reserve, whichwas demonstrated over an areaof 9630 km^2 includingreclaimed lands cover of 5366 km^2 and mangrove forest cover of 4264 km^2(Sanyal et al, 84).The entire area is covered by several backwaters which are directly feeded by tidal flow.

Apart from fishing in the fresh water and the estuarine water of the river mouths, a major portion of local inhabitants consume their livelihood by prawn cultivation and prawn seed collection of a certain specific size of PL-20 (Post Larval stage-20 days(Fig. 1). The fishing professional suffers from lack of awareness about the proper procedure of the prawn seed collection at their very early stages of growth.

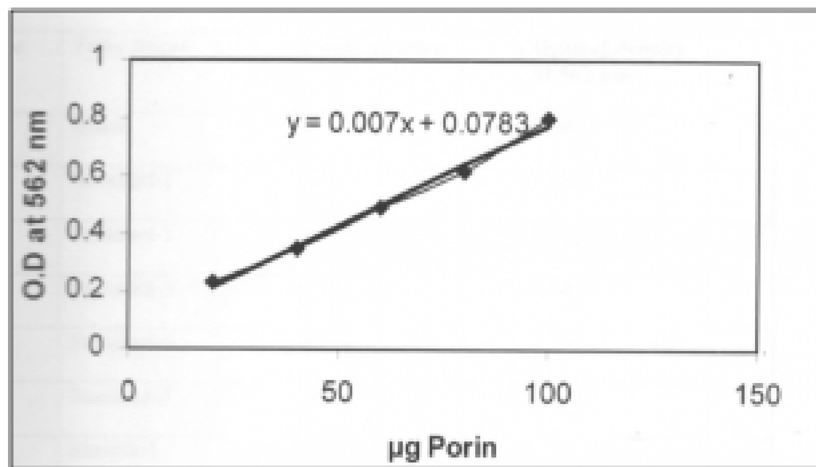


Fig. 1: Prawn Seed Collection

Adverse climate impacts and fragility of ecology often restrict rural livelihoods to mainly traditional economic activities, which mostly involve unsustainable exploitation of natural resources leading to habitat destruction and loss of biodiversity (Fig. 2 and Fig. 3). With the growing demand of Tiger Prawn (*Penaeusmonodon*) farming in Southern West Bengal, collection of Tiger Prawn seeds from the brackish estuarine water has been adopted by a large section of rural people of climate vulnerable deltaic Sundarbans. While economic return from such traditional livelihood is reducing these years, lack of eco-friendly alternative livelihood is forcing poor communities to remain engaged in this activity. Perusal of results show that untrained rural people annually segregate Tiger Prawn seeds and throw the major portion of haul to the mudflats allowing them to perish. On an average, amount of Tiger Prawn seeds are just 2.5% of the total catch. Such indiscriminate exploitation of seeds leads to drastic reduction of the species concerned and other associated communities from the estuaries.

The present paper assess on a case study from investigating the mode of seed collection from the rivers and their direct ecological impacts. Further, it estimates the economic feasibility of livelihood and suggests ecofriendly alternatives to sustain and improve livelihood and promote conservation. The study suggests that to restore ecological balance in Sundarbans ecosystem, community-ecosystem approach and adaptive management in alternative ecofriendly livelihood need to be incorporated.

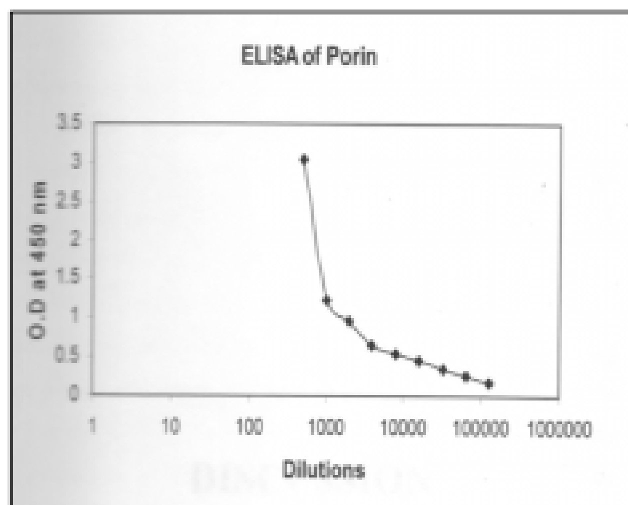


Fig. 2: Loss of Biodiversity

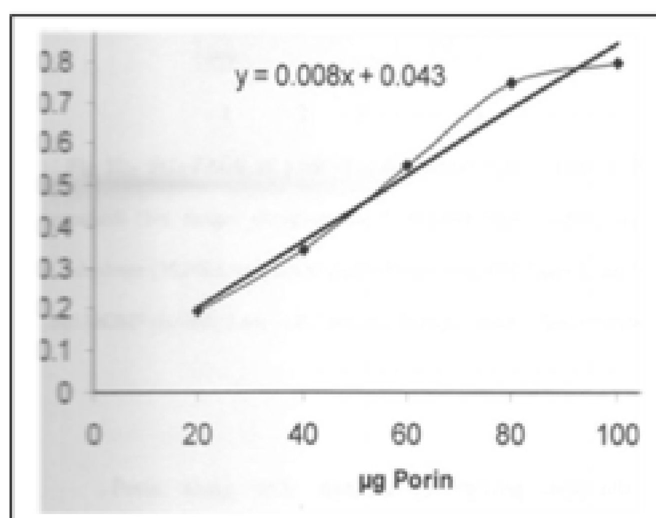
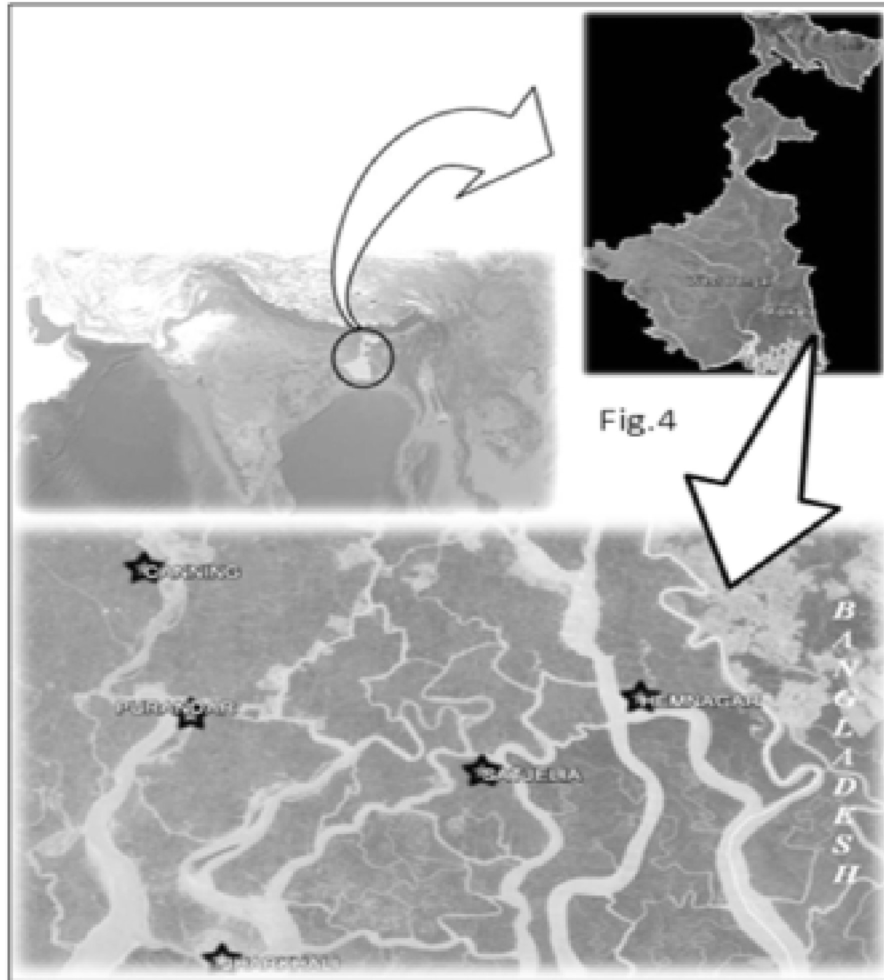


Fig. 3: Loss of Biodiversity

2. Study Site:

Sundarban is a deltaic system, distributed in two districts namely North and South 24 Parganas of West Bengal. Sundarban can be classified by two ways: it can be classifiedas western, central and eastern part (west to east) of Sundarban and on the other hand it can also be classified as outer, middle and inner estuarine system (south to north). For this study five spot namely Canning, Parandar, Jharkhali, Satjalia and Hemnagarwere selected (Map. 1). Canning (22°18'48.11"N, 88°39'29.11"E) is located at western Sundarban as well as at inner estuarine region. Parandar (22°12'1.23"N, 88°41'21.17"E) is located at western Sundarban and at middle estuarine region. Jharkhali (22° 0'37.00"N, 88°42'37.75"E) is almost located at central Sundarban portion. This is the outer estuarine region beyond which human habitation has yet been reported. Satjalia (22° 9'25.24"N, 88°52'44.89"E) is located at inner estuarine

eastern Sundarban. Hemnagar (22°12’52.07"N, 88°58’55.54"E) is located at eastern Sundarban and middle estuarine region.



Map 1: Study Sites

3. Methodology:

Prawn seeds are collected with the help of very small mesh size net by the local villagers. All the data was collected through survey in native areas of Sundarban where *meendhora*(Collection of PL 20) is widely practiced. Water sampleswere also collected from those people. Water is thrownrandomly after collection of prawn seed. Counts were taken to estimate the other seeds captured on that nets. Fishermen take only the prawn seeds and others are thrown on the estuary ridge causing huge loss of biodiversity.

After selection of study sites depending on population and feasibility, the lengths of total harvesting regionswere calculated through spot measurement and verified by remote sensing tool. River banksare marked for each location, showing in the following map group (Map 2, Map 3, Map 4, Map 5 and Map 6).

Map 2: Jharkhali

Map 3: Hemnagar

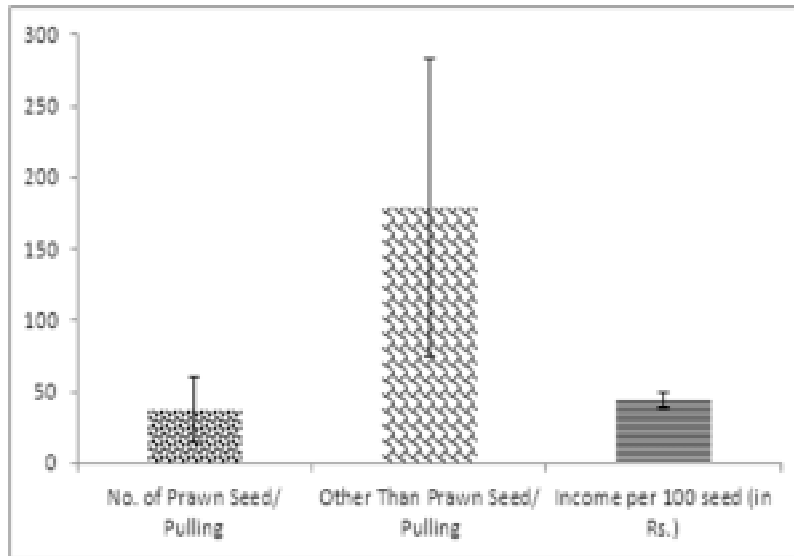
Map 4: Parandar

Map 5: Satjalia

Map 6: Canning

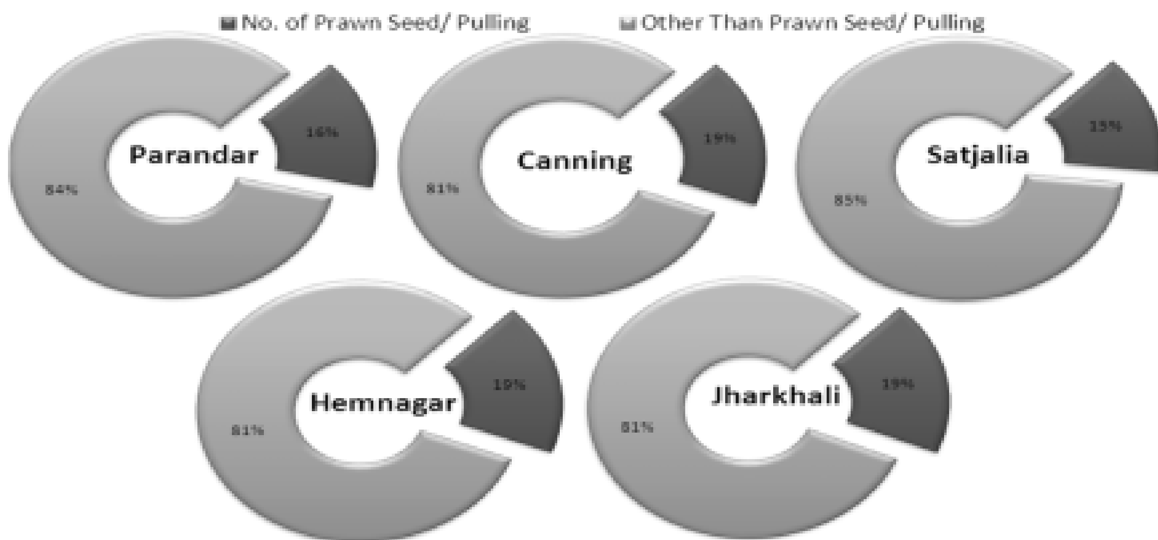
4. Result and Discussion:

Afteranalyzingdata of all the study sites, it can be found that average wastage of other species than individual prawn seed is much higher in numberwhich is almost four times (Graph1).Though, price of PL-20 varies place to place, nowhere the overall income through seed collection is satisfactory.



Graph 1: Average per pull

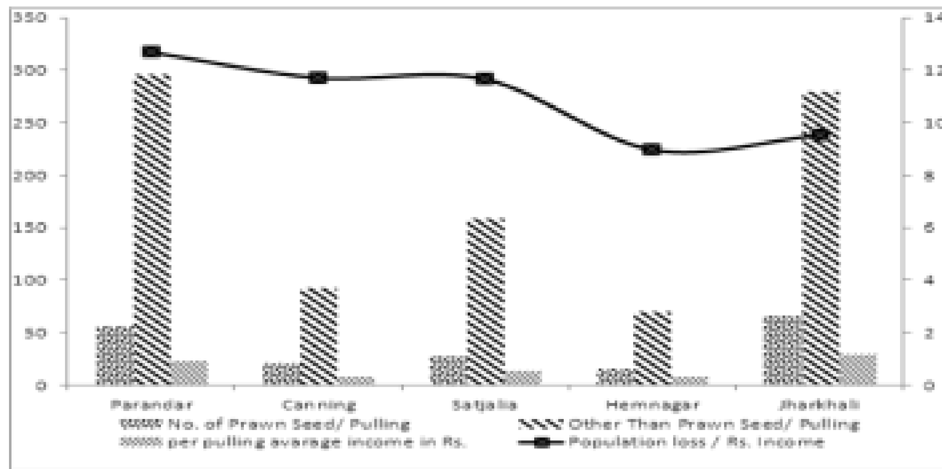
Site wise, average number of prawn seed and average number of others, captured per pulling is shown in Graph 2 which shows % of loss is almost same in each site.



Graph 2: Catchment percentage in study sites per pull

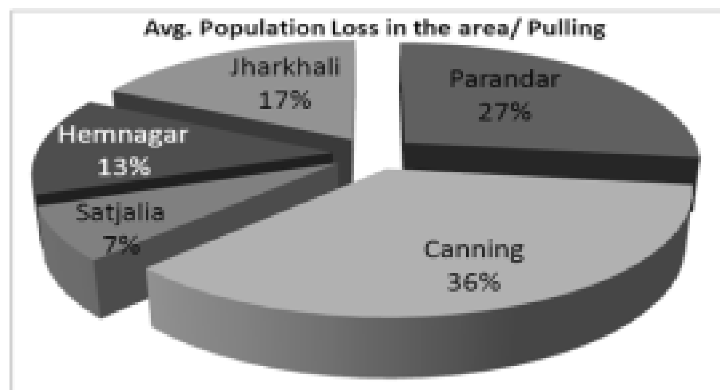
 ISBN: 978-81-928924-3-6

Result also shows (Graph 3) that, the catchment capacity of PL-20 pulling is much higher in Parandar and Jharkhali. According to Baidya and Choudhury (1984), western Sundarban has higher ichthyoplankton population than its eastern part. Parandar is in western site, so it is quite natural to get much seed here, and for Jharkhali (outer region), catchment is low because of more sea water influx. Canning is situated at inner estuarine region, thus gets less tidal influx which carries less population density, whereas, at Hemnagar, catchment is low because of its location (eastern). In case of Satjalia the above mentioned parameters are moderate. But it can be seen that the biodiversity loss per unit income generation is almost same throughout Sundarban, for generating INR 1 almost 10 to 12 individuals are destroyed.



Graph 3: Catchment capacity in study sites

If the catchment length pulled only one time, the loss of biodiversity due to seed collection is much less in eastern side, around 2000/pulling. Because of finding of less seeds (22/pulling), collectors have to move for their alternate income generation. In case of western side biodiversity loss is quite higher (Graph 4) because of good population density. Over exploitation gives good income at first stage but its decrease gradually and collectors have to suffer afterwards.



Graph 4: Population Loss for Sites

5. Conclusion:

After getting the proper knowledge and awareness about the prawn seed collection, fish culture in a scientific way, theimportance of biodiversity, can protect and maintain the environment. There will be a lot of employment and the rate of production at every sector will be increase naturally.

References:

1. Baidya, A., A. Choudhury.. Copepod Component of Zooplankton in a tidal creek (Chemaguri) of Hooghly estuary, Sagar Island, Sundarbans, West Bengal. *Environment & Ecology*, 1984, Vol. 2(3): 162-167.
2. Central Inland Fisheries Institute. *1992-93 Annual Report*. CIFI, Calcutta.
3. Chakrabarti N.M., A. K. Roy, A. Ghosh.. An improved growth, survival and yield in biculture of brackish water fish and prawn through multiple stocking and repeated harvesting. *The Journal of the Indian Society of Coastal Agricultural Research*,1984, 2 (1): 33-40
4. Government of India. 1990. *Conservation of Mangroves in India*. Ministry of Environment and Forests, New Delhi.
5. IUCN..*Marine Protected Areas Needs in the South Asian Seas Region*.,1989, Volume2, India.
6. Mandal A. K..A study on the haematozoa of fishes of commercial importance from India.*Technical Monograph No. 9*.Zoological Survey of India, Calcutta.,1984, 1-51.
7. Mandal A. K., R. K. Ghosh. 1989. *Sundarbans: A socio bio-ecological study*. Bookland Private Ltd., Calcutta.
8. Sanyal P., L. K. Banerjee, M. K. Choudhury.. Dancing Mangals of India Sundarbans. *The Journal of the Indian Society of Coastal Agricultural Research*, 1984, Vol.2 (1): 10-16.
9. Sarkar S. K., B. N. Singh, A. Choudhury.. The Ecology of copepods from Hooghly Estuary, West Bengal, India, Mahasagar. *Bulletin of the National Institute of Oceanography*.,1986, Vol.19 (2): 103-112.
10. Sundarbans Development Board. 1992. *Annual report*. Government of West Bengal, Calcutta.

Chapter 13

Assessment of plastics as refuge for aquatic organisms in urban ponds

***Amritangshu Roy**
R B B High (H.S.) School, Garhjoypur, Purulia, West Bengal, India
***Corresponding author:** amritangshuroy@gmail.com

Abstract

1. Introduction:

Ponds are defined as Water bodies between 1 m^2 and 2 ha in area, which may be permanent or seasonal, including both manmade and natural water bodies (Biggs et al., 1998, 2005; DeMeester et al., 2005). The urban ponds are potential site for conservation and maintenance of biological diversity (Biggs et al., 1998; DeMeester et al., 2005; Dudgeon et al., 2008a, 2008b). Apart from being the ecosystems services delivered by the urban ponds, the presence of numerous different aquatic organisms indicates the potential for biodiversity conservation (Dudgeon et al., 2006; Gledhill et al., 2008; Davis et al., 2008b; Oertli et al., 2009). Owing to their location in urban areas, these habitats are exposed to environmental pollutants form varied sources (Gobel et al., 2007). Pollution of urban ponds is a concern, globally, since it affects the ecosystem services and hinders the natural biota inhabiting the ponds. As a consequence of such stress owing to pollutants, in many instance a retrogressive successional mechanism becomes predominant to bring a permanent shift to a bog and continued change interrestrial habitat condition (Biggs et al., 2005). The degradation of the urban pond ecosystem may be due to organic pollutant load leading to eutrophication or else deposition of non degradable pollutants like plastics and metals (Gobel et al., 2007; Scheffer et al., 2008). These calls for an exploration of the effects of such hazardous pollutants that are entering the pond system through several ways including domestic house hold floating debris (Brich & McCaskie, 1995). In this context a preliminary assessment was made through a field study using selected urban ponds of Burdwan Municipal area as model habitats.

The results of the study are expected to highlight the possible effects of hazardous waste in aquatic communities and thereby information needs to mitigate such problem. It would also help to prioritize the management plans (Oertli et al., 2009) required to prevent degradation of restoration strategies.

2. Materials and Methods

The study area considered was limited to selected wards of the Municipality and included eight different ponds within the area concerned. The ponds were chosen after a pilot observation

on their location nearer to human settlement and that area expected to absorb household floating plastic debris (FPD) through different path.

In each pond a visual survey was followed by collection of the plastic debris that remains floating on the surface using a long handle catcher or lander. The associated macro-invertebrates were counted and recorded on the spot and the floating plastic along with the adhered organism were placed in plastic bag and brought to the laboratory. In the laboratory the macro-vertebrates were further segregated, identified, counted and recorded (Edmondson, 1963). The plastic debris were classified as large, medium and small based on the length dimension and biomass. Water of the pond was also collected for evaluating the chlorophyll *a* content that was used as surrogate of productivity status of the ponds. The chlorophyll *a* content (APHA, 1998) of selected plastics obtained from the pond were also measured to comment on the autotrophic base of the floating debris. A t-test (Zar, 1999) was applied on the data on chlorophyll content between the FPD and the pond water to comment on the difference if any.

3. Results and Discussion

The number of floating plastic debris in nth ponds varied in shape size and biomass. All the ponds were noted to be positive for the presence of floating plastic debris (FPD) and acted as substratum for different group of macro-invertebrates like chironomid larvae, pulmonate snails, dytiscid beetles, dragonfly nymphs along with considerable amount of chlorophyll apparent from the greenish tinge on the surface of the debris (Tables 1 and 2). The ratio to predator is shown in Fig 1. In only ten samples of FPD, the prey to predator ratio remained more than one while in most instances the prey or predator taxa were present only. The low occurrence of prey predator ratio is an indicator that the FPD were possible used as refuge by the preys or the predators. The FPD could possibly be used as safe place for these macro-invertebrates to avoid competition for space and resource. The plastic debris were chiefly of thermocol origin and contained high deposition of algae as reflected from chlorophyll content. The chlorophyll content of the pond water and the thermocol debris were significantly different revealed by paired sample t-test ($t = 2.86$; $df = 6$, $p < 0.01$) (Fig 2). The presence of chlorophyll and the chironomid larvae suggest that the FPD could accumulate small particulate organic matter such that it transformed into congenial habitat for the growth of algae and the detritivores and collectors. The result of the present survey is indicative of the fact that the floating debris of allocthonous origin is being utilized by macro-invertebrates as refuge and can possible influence the species interaction in the ponds. In long run these may permanently affect the biological diversity of the urban ponds. Thus immediate action may be taken to ensure strict legislation in reducing the entry of FPD in the ponds that are potentially rich in maintenance for biological diversity in urban conditions.

 ISBN: 978-81-928924-3-6

Table 1: The number of floating plastic debris (FPD) and the total number of macroinvertebrates and chlorophyll content of pond water and FPD (in mg/L).

		FPD	Chironomid larvae	Dytiscid beetle	*Lymnaea*	*Bellamya*	*Indoplanorbis*	Odonate Nymph	*Tubifex*	*Gobbio*	Chlorophyll content	chlorophyll in plastics
B.Ed College	POND 1	1.1	4	0	0	0	0	0	0	0	0.1	
		1.2	0	2	0	0	0	0	0	0	0.1	
		1.3	3	0	0	0	0	0	0	0	0.1	
		1.4	0	0	10	0	0	0	0	0	0.03	
		1.5	0	0	5	0	0	0	0	0	0.03	
		1.6	4	0	2	0	0	0	0	0	0.03	
Bharathi School 2	POND 2	2.1	2	0	2	0	0	0	0	0	0.09	0.453
		2.2	2	0	0	0	0	0	0	0	0.09	0.453
		2.3	0	0	0	1	0	0	0	0	0.09	0.453
		2.4	0	0	6	0	0	0	0	0	1.28	0.453
		2.5	0	0	12	0	0	0	0	0	1.28	0.453
		2.6	4	0	6	1	0	0	0	0	1.28	0.453
Borehat	POND 3	3.1	6	0	0	0	0	0	0	0	0.04	0.357
		3.2	0	0	4	0	0	0	0	0	0.04	0.357
		3.3	6	0	0	0	0	0	0	0	0.04	0.357
		3.4	3	2	0	0	0	0	0	0	0.04	0.357
		3.5	34	0	0	0	0	0	0	0	0.04	0.357
		3.6	0	0	0	1	0	0	0	0	0.04	0.357
Lakurdi	POND 4	4.1	6	0	2	0	0	0	0	0	0.02	0.352
		4.2	15	0	0	0	0	0	0	0	0.02	0.352
		4.3	14	0	0	0	0	0	0	0	0.02	0.352
		4.4	6	0	0	0	0	0	0	0	0.02	0.352
		4.5	4	0	2	0	0	0	0	0	0.02	0.352
		4.6	6	0	0	0	0	0	0	0	0.02	0.352
Lisarpar	POND 5	5.1	3	0	1	0	0	0	0	0	0.61	0.395
		5.2	2	3	1	0	0	0	0	0	0.61	0.395
		5.3	4	0	0	0	0	0	0	0	0.61	0.395
		5.4	0	1	3	0	0	0	0	0	0.61	0.395
		5.5	4	0	0	0	0	0	0	0	0.61	0.395
		5.6	3	2	0	0	0	0	0	0	0.61	0.395

Site	Pond	FPD	Chironomid larvae	Dytiscid beetle	*Lymnaea*	*Bellamya*	*Indoplanorbis*	Odonate Nymph	*Tubifex*	*Gabbia*	*Chlorophyll content*	*chlorophyll in plastics*
Ranisayar	POND 6	6.1	12	1	1	0	1	0	0	0	0.03	0.315
		6.2	6	0	0	0	1	0	0	0	0.03	0.315
		6.3	2	0	2	0	1	0	0	0	0.03	0.315
		6.4	0	1	0	0	0	0	0	0	0.03	0.315
		6.5	2	0	4	0	0	0	0	0	0.03	0.315
		6.6	8	0	0	0	0	0	0	0	0.03	0.315
		6.7	6	0	0	0	0	0	0	0	0.03	0.315
		6.8	0	0	0	0	0	1	1	0	0.03	0.315
		6.9	4	0	0	0	0	0	0	0	0.03	0.315
		6.10	2	0	0	4	0	0	0	0	0.03	0.315
		6.11	0	0	0	4	0	0	0	0	0.03	0.315
Sibmandir	POND 7	7.1	34	0	0	0	0	0	0	0	0.12	0.379
		7.2	36	0	0	0	0	0	0	0	0.12	0.379
		7.3	13	0	0	0	0	0	0	0	0.12	0.379
		7.4	0	0	0	0	0	0	0	2	0.12	0.379
		7.5	3	0	1	0	0	0	0	0	0.12	0.379
Shyamsayar	POND8	8.1	1	1	0	0	0	0	0	0	0.22	0.379
		8.2	0	0	0	0	0	1	1	0	0.22	
		8.3	1	1	0	0	0	0	1	0	0.22	
		8.4	0	0	0	0	0	0	0	0	0.22	
		8.5	18	0	0	0	0	1	0	0	0.22	

Table 2. The macro invertebrate richness of the floating plastic debris collected from different ponds (n=8). Predators are marked in BOLD.

Table 3. Correlation Matrix based on abundance of different macro invertebrates collected from the floating plastic debris.

	A	B	C	D	E	F	G
B	-0.162						
C	-0.247	-0.124					
D	-0.143	-0.112	-0.076				
E	0.033	0.022	-0.025	-0.067			
F	0.013	-0.104	-0.124	-0.067	-0.063		
G	-0.155	0.022	-0.124	-0.067	-0.063	0.646	
H	-0.093	-0.059	-0.070	-0.038	-0.035	-0.035	-0.035

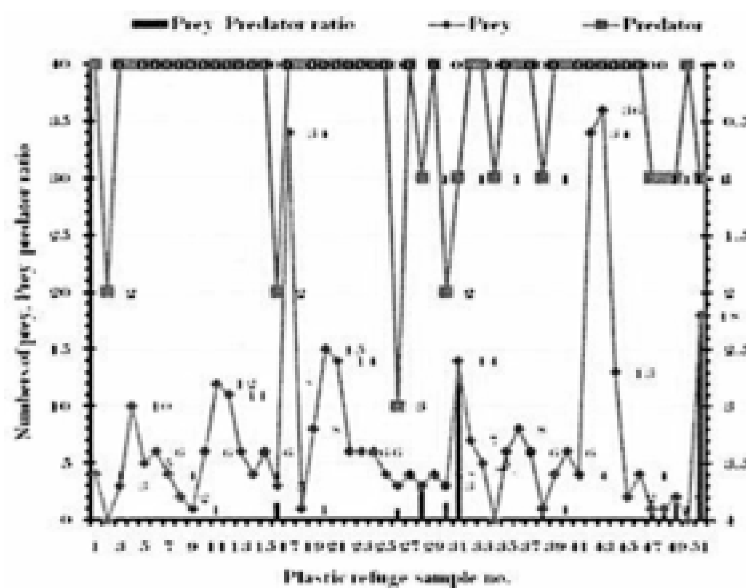


Fig.1: Prey-predators ratio in the floating plastic debris (FPD) from the right ponds. (n=51)

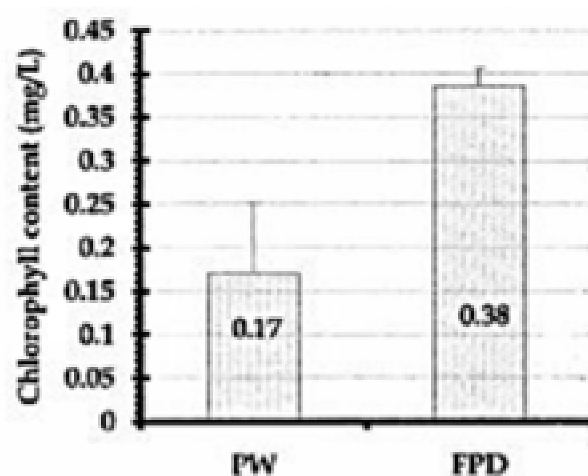


Fig. 2: Chlorophyll content in Pond water (PW) and floating plastic debris (FPD) samples

References

1. APHA, AWWA, WPCP, Standard methods for the examination of water and waste water., American Public Health Association, New York, USA.1998, 19th edition.
2. Biggs, J., Fox, G., Nicolet, P., Walker, D., Whitfield, M. and Williams, P.A guide to the methods of the National Pond survey. Oxford, Pond Action.1998.
3. Biggs. J.,Williams, P., Whitfield, M., Nicolet, P. and Weatherby, A. "15 years of pond assessment in Britain: results and lessons learned from the work of Pond Conservation." aquatic Conservation-Marine and Freshwater Ecosystems, 2005, 15(6): 693-714
4. Birch.S. and McCaskie, J. "Shallow urban lakes: a challenge for lake management." Hydrobiologia, 1999, 395: 365-377
5. Davies, B., Biggs, J., Williams, P., Whitfiels, M., Nicolet, P., Sear, D., Bray, S. and Maund, S."Comparative biodiversity of aquatic habitats in the European agricultural landscape." Agriculture Ecosystems & Environment, 2008a, 125(1-4): 1-8.
6. Davies, B.R., Biggs, J., Williams, P.J., Lee, J. T. and Thompson, S. "A comparison of the catchment sizes of rivers, streams, ponds, ditches and lakes: implications for protecting aquatic biodiversity in an agricultural landscape." Hydrobiologia, 2008b, 597:7-17.
7. De Meester, L., Declerck, S., Stoks, R., Louette, G., Van de Meutter, F., De Bie, T., Michels, E. and Brendonck, L. " Ponds and pools as model systems in conservation biology. ecology and evolutionary biology." Aquatic Conservation-Marine and Freshwater Ecosystems, 2005, 15(6): 715-725.
8. Dudgeon, d., Arthingtion, A.H., Gessner, M.O., Kawabata, Z.I., Knowler, D.J., Leveque, c., Naiman, R.J., Peieur-Richard, A.H., Soto, D., Stiassny, M.L.J. and Sullivan, c.A. "Freshwater biodiversity: importance, threats , status and conservation challenges." Biological reviews, 2006, 81(2): 163-182.
9. Edmondson, W.T. "Fresh-water Bilolgy". John Wiley Inc. Indian Repring, IBPSS, New Delhi, India.1963.
10. Gledhill, D.G., James, P. and Davies, D.H. "Pond density as a determination of aquatic species richness in an urban landscape." Landscape Ecology, 2008, 23(10) 1219-1230.
11. Gobel, P., Dierkes, C. and Coldewey, W.C. "Strom water runoff concerntation magere for urbnan areas." Journal of contaminant Hydrology, 2007, 91(1-2): 26-42.
12. Oertli, B,.careghino, R., Hull, A. and Miracle, R. "Pond conservation from science to practice." Hydrobiologia, 2009, 634(1):1-9
13. Scheffer, M., van Geest, G.J., Zimmer, K., Jeppesen, E., Sondergaard, M., Butler, M.G. Handson, M.A., Declerck, s. and De messter, L. "Small habitat size and isolation can promote species richness: scond –order effects on biodiversity in shallow lakes and ponds." Oikos, 2006, 112(1):227-231.
14. Zar, J.H. Bostatistical analysis IV ed. Person education Singapore Pte. Ltd. (Indian branch), New Delhi India.1999.

Chapter 14

Isolation of Porin from *Shigella dysenteriae* type1 to establish it as a Vaccine component

***Deb SinghaRoy**
Alkem Laboratories ltd., Sikkim, India
***Corresponding author:** debsingharoy1988@gmail.com/
deb.singharoy29604@alkem.co.in

Abstract

Shigella spp. Is a gram negative bacteria cause bacillary dysentery or shigellosis. These are resident of intestinal tract of human, apes and monkeys. They are not much affected by stomach acidity. Herein we describe the isolation of Porin protein of *Shigella dysenteriae* type 1 and establishment of porin as a vaccine. Porins are trans-membrane proteins consist of three beta barrel protins. Unlike other membrane transport proteins porins are large enough to allow passive diffusion; they act as channels that are specific to different type of molecules. Porins are immunogenic without the addition of exogenous adjuvants and are known to be able to augment the humoral response to otherwise poorly immunogenic substances, for example polysaccharides and peptides. These proteins are of particular interest because they have been characterized as potent adjuvants and have great potential as a novel component of vaccines. Porin of *S.dysenteriae* type 1 has been found to surface exposed, strongly immunogenic and antigenically related among Shigella spp (Roy et al 1994). These features make this protein attractive as an adjuvant for use in vaccine formulation against shigellosis.

Keyword

Shigellosis, porin, vaccine

1. Introduction

*Shigella spp.*is a gram-negative bacteria causes bacillary dysentery or shigellosis. These are the resident of the intestinal tract of human, apes and monkeys, they are not much affected by stomach acidity. As we can find the general property of *Shigella spp.* – they are rod shaped, non-spore forming, non-motile, intracellular, facultative anaerobe. It multiplies within the epithelial cells of colon (Maurelli and Sansonetti, 1988).

Shigellosis is a major form of Bacillary dysentery, caused by infection with *Shigella*organisms.In poor countries, *Shigella*-caused dysenteriae is endemic and causes an estimated 163 million illness episodes annually and more than one million deaths (Keusch, 1982; Hale, 1991). Only 10 to 100 bacteria can cause shigellosis in human. There are several

species of *Shigella* that can cause different type of shigellosis in human – they are *Shigella dysenteriae, S.flexneri*, *S.sonnei, S, boydii*etc.

Shigellosis is a major public health problem and it is one of the most occurring causes of child death in developing countries. Increased incidences of antibiotic resistance in Shigella spp. constitute a major concern. High frequency of resistance of *Shigella flexneri*to many of the first line antimicrobial agents(multi drug resistant) have been reported in recent years from Kolkata (Dutta et al., 2003).

Infection occurs in several steps (1) they proliferate to immense numbers in the small intestine, but the primary site of disease is large intestine. (2) *Shigella* attaches to the epithelial cell of large intestine, then ente the epithelial cell (LaBrec et al., 1964). (3) Multiplication occurs inside the cell (Sansonetti et al., 1986). (4) Shigella invades neighboring epithelial cells, thus avoiding immune defenses. (5) An abscess forms as epithelial cells are killed by the infection. (6) The total epithelial cell lining is destroyed. (7) Cell death leads to a strong inflammatory response and forms ulceration.

The outer membrane of gram-negative bacteria consists of mainly phospholipids, proteins and lipopolysaccharides. Nearly half or entire outer membrane is made of protein. These include OmpA, murein, lipoproteins and porins. Major beta barel proteins in gram-negative cell outer membranes group as dimers or trimmers that form transmembrane channels allowing transport of certain molecules into the cells. Unlike other membrane transport proteins, porins are large enough to allow passive diffusion, i.e., they act as channels that are specific to different types of molecules.

Porins are tubes with a diameter of about 1 nm which are filled with water. Nonspecific porins allow the diffusion of ions and molecules up to a molecular weight of 600 (Benz et al., 1985; Hancock, 1987). The diffusion speed depends on both the difference of concentration in the periplasm and outside and the molecular weight of the solute.

The construction principle of porins is the same irrespective of their type: a chain of 300 – 420 amino acids folds to an antiparallel beta-barrel of 16 or 18 strands (Rosenbusch, 1974; van der Ley et al., 1986). The wall of the pore has a thickness of one amino acid only. On the side of the barrel facing the periplasm the beta strands are connected by short loops or turns. On the other side the loops directed to the environment are large and variable. The loop connecting beta strands 5 and 6 is of special importance: it is folded into the barrel and constricts the cross section. At the narrowest point there are some ionizable amino acids. The filter properties of the pore are defined at this point.

This principle is found in other nonspecific porins too, although there is no sequence homology. In the frames below, the starting view is from the periplasm through the pores to the outside.

Porins are inserted in the outer membrane as trimmers. Amino and carboxy termini of the single molecules face the threefold symmetry axis of the complex. As found in other transmembrane proteins there are two belts of aromatic amino acids pointed to the surfaces of the membrane. Between the belts the surface of the barrels is composed mainly from hydrophobic amino acids. The belts are placed in a distance of approx. 25Å corresponding to

the thickness of the outer membrane. The loops at the outer face narrowed down the opening of the pores. One loop is positioned in a way to line the opening of a neighboring pore. This 'domain swapping' stabilizes the quarternary structure.

Porins are immunogenic without the addition of exogenous adjuvants and are known to be able to augment the humoral response to otherwise poorly immunogenic substances, for example, polysaccharides and peptides. These proteins are of particular interest because they have been characterized as potent adjuvants and have great potential as a novel component of vaccines. Porin of *S. dysenteriae* type 1 has been found to be surface exposed, strongly immunogenic and antigenically related among *Shigella spp*(Roy et al., 1994). These features make the protein attractive as an adjuvant for use in vaccine formation against shigellosis.

2. Materials and Methods

2.1. Preparing Bacterial Culture

2.1.1. Materials:

1. Bacteria Shigella dysenteriae type 1, isolate no. A020332 was obtained from International Centre for Diarrheal Research (ICDDR), Bangladesh. For experiment a Shigella dysenteriae type 1 stab was taken.
2. Nutrient Broth
3. Xylose lysine desoxycholate agar (XLDA)
4. Triple sugar iron slant.
5. Tryptic Soy broth (1 litre)

2.1.2. Method:

1. Activating Bacteria from stab: Shigella dysenteriae type 1 taken from the stab and inoculated in a small amount of nutrient broth in a test tube for activating the bacteria. The inoculated test tube for activating the bacteria. The inoculated test tube kept in incubator at 37°C with shaking for 6 to 8 hours.
2. XLDA plate: Isolated culture of Shigella dysenteriae type 1 was performed in aa selective medium i.e. XLD medium.
3. The bacterium was then putted in a TSI slant and incubated for 20 to 22 hours. The acid Yellow butt and alkaline slant showed the presence of Shigella spp. Specifically.
4. 50ml Seed culture: Isolated colony taken from the XLDA plate and inoculated in 50ml of Tryptic Soy broth (TSB) and kept it in 37°C with shaking overnight.
5. 4 litre culture: 50ml seed culture was used as inoculum for 4 litre TSB medium. S. dysenteriae was cultured in 4 litre TSB at 37°C with shaking for 16 hours.

2.2. Isolation of OMP:

2.2.1. Materials:

1. Phosphate buffer saline (pH 7.2) – 1000ml
2. 1% sodium lauroylsarcosine in PBS – 50 ml
3. 10 mMTris- 2% SDS (pH 7.7) – 100ml
4. Extraction buffer (pH 7.7) – 2000ml

2.2.2. Methods:

1. 4 litre culture of Shigella dysenteriae was centrifuged at 10,000 x g for 10 minutes at 4°C.

2. The pellet was washed and suspended with 50ml PBS and collected in a beaker.
3. The cells were disputed in a ultrasonic disintegrator with 40 pulse of 10 seconds each. In this process the cells were disrupted and the cell membrane got separated from the cytoplasm.
4. After ultrasonication the suspension was centrifuged at 10,000 x g for 10 minutes to separate the undisrupted cells from the suspension.
5. The supernatant was taken and centrifuged at 1,00,000 x g for 1 hour at 4°C to isolate the envelope fraction.
6. The pellet was suspended in 50ml of 1% sodium lauroylsarcosine (Sigma Chemical co., St.Louis, Moussorie) in PBS and stirred gently at 25°C for 30 minutes to solubilize the inner membrane (Filip et al., 1973).
7. Suspension was centrifuged at 1,00,000 x g for 1 hour. After centrifugation the pellet was taken. The pellet contained the OMP fraction.

2.3. Isolation of Major outer membrane protein (MOMP):

2.3.1. Methods:

1. The pellet was washed with 10 mMTris – 2% SDS buffer and collected in a conical flask.
2. The suspension was incubated at 37°C for 1 hour with shaking.
3. The suspension was centrifuged at 1,00,000 x g for 1 h at 4°C.
4. Pellet was dissolved in 2% SDS and incubated at 37°C for 1 h with shaking.
5. The suspension was centrifuged at 1,00,000 x g for 30 minutes.
6. Pellet was dissolved in 6ml Extraction buffer and incubated for 2h at 37°C with shaking.
7. OMP-EB mixture was centrifuged at 1,00,000 x g for 1h at 25°C.
8. The supernatant was collected into a 15ml tube and the pellet is discarded.

2.4. Size exclusion chromatography:

The extraction buffer was allowed to enter the column completely and the MOMP fraction was loaded on the column. The flow rate of the column was adjusted to 0.5ml/min using a BIO-Rad ECONO pump. 60 different fractions are collected and the absorbance of each fraction recorded at 280 nm. The peak MOMP fractions were pooled and electrophoresed.

2.5. Defining the concentration of porin by Bicinconinic acid prtein assay:

- Protein Reagent: 1 part 4% copper sulphatepentahydrate with 50 part bicinconinic acid.
- Standard solution: 1mg/ml bovine serum albumin.
- The samples were mixed well and incubated at 37°C for 30 minutes and absorbance was recorded at 562nm.

2.6.Qualitative determination of porin by SDS-PAGE:

The SDS-PAGE was performed in proper way. The gel was run at 70 volt till the tracker dye is approximate 1 cm above the end of the glass plates.

The Gel was removed and placed in a container containing Coomassie Brilliant Blue stain in a way that the gel must stay in total submerged condition. Gel was stained for 1h, with gentle shaking. Then the gel was distained in distaining solution until the background stain was removed.

The molecular weight of protein was determined by comparing them with the molecular weight markers.

2.7. Dialysis of Porin:

Porin was dialyzed against dialysis buffer in dialysis bag. Dialysis continued for 3 days with two changes of dialysis buffer each day. After dialysis the concentration of dialyzed sample was estimated by Bicinconinic acid protein assay method.

2.8. Injecting in Mice:

C57BL/6 mice were obtained from National Centre for Laboratory Animal Sciences, National Institution of Nutrition, Jamia-Osmania, Hyderabad, bred and reared in the animal care facility of National Institute of Cholera and Enteric Diseases, Kolkata. The Mice were housed in groups of five and given food and water ad libitium. The experiment with animals was conducted in accordance with the Animal Ethical Committee guidelines of National Institute of Cholera and Enteric Diseases, Kolkata. The mice were injected intraperitoneally with 30µg of purified MOMP emulsified with Freund complete adjuvant (Gibco). This was followed by two booster doses at an interval of 10 days. Antisera were collected by puncturing of the supraorbital plexus.

2.9. Western Blotting:

Western Blotting was performed to determine the antigenicity of Porin.

1. Electrophoresed porin of Shigella spp.was transferred to a nitrocellulose membrane at 100 V for 1h 20min in a Bio-Rad mini transblot chamber in transfer buffer.
2. After the transfer, membranes were blocked with blocking buffer overnight.
3. Then the membranes were incubated for 3 h with mice anti-sera (diluted 1:500 in TBS/T with 5% BSA, 7ml).
4. After incubation antigen-antibody complexes were detected with secondary antibody (alkaline phosphatase conjugated anti-mouse igG) for 1 hour.
5. The nitrocellulose membranes were washed thoroughly in TBS/T and developed with 0.3 mg of p-nitrobluetetrazolium chloride and 0.15mg of 5-bromo-4-cloro-3-indoyl phosphate per ml in carbonate buffer (pH 9.8) containing 0.1M $NaHCO_3$ and 1.0mM $MgCl_2$ to visualize the bands.

2.10. ELISA:

96 well micro-enzyme-linked immunosorbant assay (ELISA) plates (Gibco) were coated with either 100µl of purified MOMP (10µl per well in 50mM Tris-HCl, pH 8.1). After overnight incubation at 4°C, the wells were blocked with 250 µl of 2% BSA. The plates were incubated for 2h at room temperature (RT). After blocking, 100 µl of anti-MOMP antibody previously absorbed with LPS of S.dysenteriae type 1 wasadded to the wells at different dilutios, and the plates were incubated for 1 h at room temperature. After incubation, the wells were washed with PBS (pH 7.2) containing 0.05% (vol/vol) Tween 20. 100 µl of peroxidase-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove) at a 1:2,000dilution was then added and the plates were incubated for 45 min at room temperature. The wells wereagain washed repeatedly and 100 µl of TMB substrate solution(BD Biosciences Pharmingen) previously prepared by mixing equal volume of substrate reagent A(containing

hydrogen peroxide in buffered solution) with substrate reagent B(containing TMB in an organic solvent) was added to each well. The plate was incubated for 30mins in the dark. The reaction was stopped with 50 µl per well of 1 M phosphoric acid. OD_{450} of each well was measured by a microplate reader (Model 550 Microplate Reader, BioRad, Japan).

3. Results and Disscussion:

3.1. Results:

The purified porin obtained from column chromatography on Sephacryl S-200 HR column (1.6 by 90 cm) was estimated by bicinchoninic acid method.

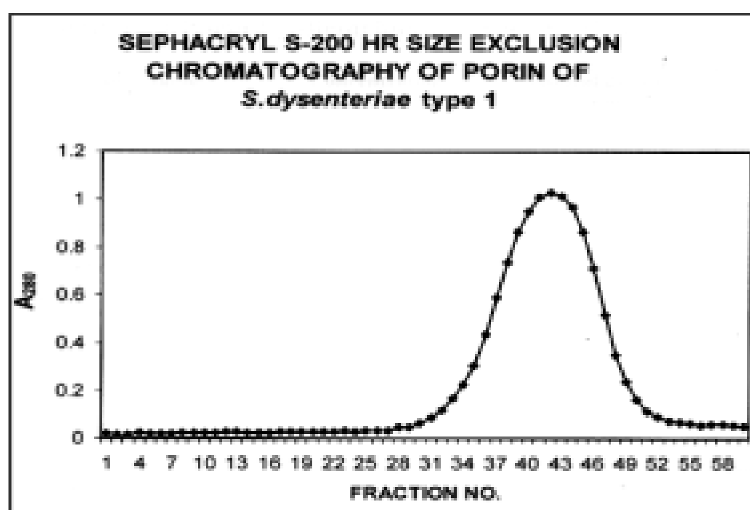


Fig1: Profile of porin of S.dysenteriae type 1 eluted through a Sephacryl S-200HR column

3.1.1 Estimation of Porin by Bicinchoninic Acid Method:

Tube No.	Tube Name	Concentration	Optical Density at 562nm
1	Blank	**0**	**0.000**
2	Standard- 1	**20**	**0.232**
3	Standard- 2	**40**	**0.350**
4	Standard- 3	**60**	**0.494**
5	Standard- 4	**80**	**0.615**
6	Standard- 5	**100**	**0.799**
7	Sample-1	**-**	**0.473**
8	Sample-2	**-**	**0.310**

Concentration was estimated to be 0.65 mg/ml using BSA as standard.

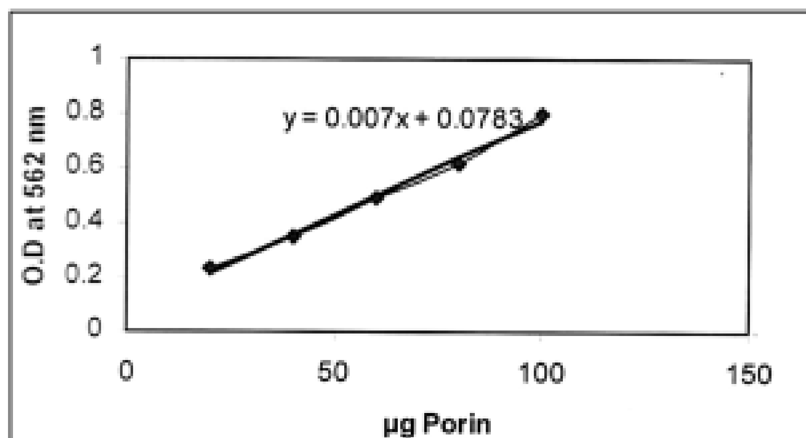


Fig 2: Estimation of porin by bicinchoninic acid method.

3.1.2 Estimation of Porin by Bicinchoninic Acid Method after Dialysis:-

Tube No.	Tube Name	Concentration	Optical Density at 562nm
1	Blank	**0**	**0.000**
2	Standard- 1	**20**	**0.195**
3	Standard- 2	**40**	**0.341**
4	Standard- 3	**60**	**0.545**
5	Standard- 4	**80**	**0.745**
6	Standard- 5	**100**	**0.795**
7	Sample-1	**-**	**0.382**
8	Sample-2	**-**	**0.261**

Concentration was estimated to be 0.5 mg/ml sing BSA as standard.

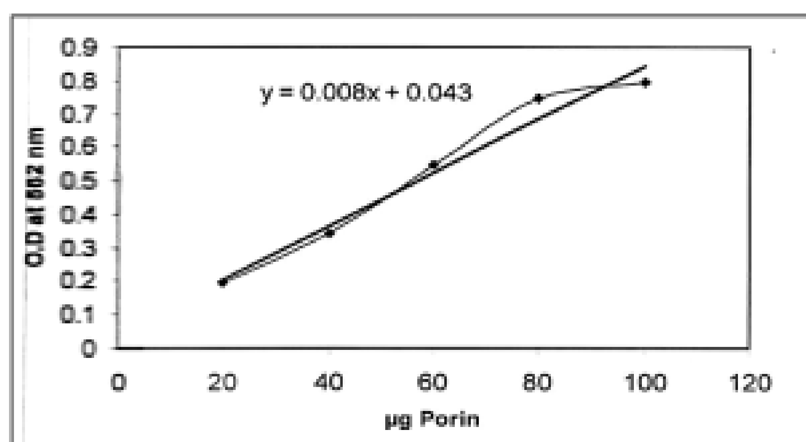


Fig 3: Estimation of porin by bicnchoninic acid method after dialysis.

3.1.3 SDS-PAGE of porin of S.dysenteriae type 1:

Porin along with standard of varying molecular weights was electrophoresed. Unboiled porin showed an oligometric band of 78,000 Dalton. However when it was boiled at 100°C for 5 mins, prior to electrophoresis, it produced a monomeric band of 38,000 Dalton.

3.1.4 Western Blot Analysis of Porin:

Western blot analysis revealed that anti-sera at 1:500 dilution, obtained from porin immunized C57BL/6 mice, reacted strongly and specifically with porin of both S.dysenteriae type 1 and S.flexneri type 2a. This indicates that porin is immunogenic and antigenically related among Shigella spp.

3.1.5 ELISA of Porin:

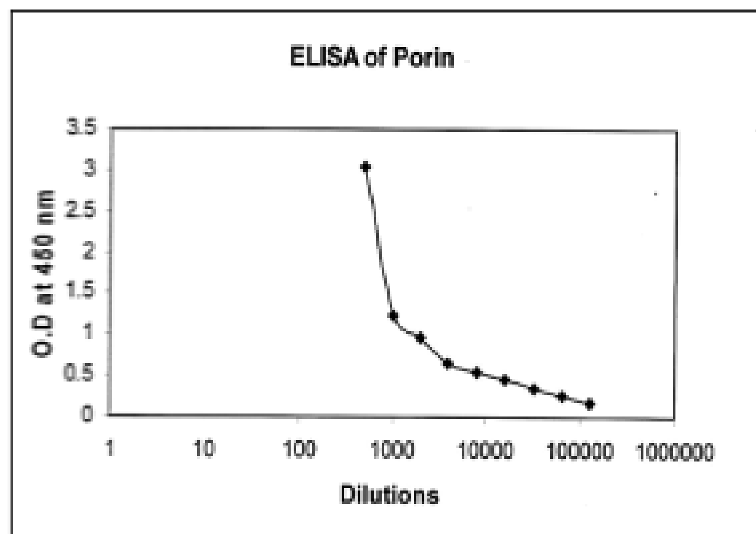


Fig 4: Detection of porin specific antibody by direct ELISA

The titer of porin specific antibody generated after immunization is very high as it was detected even at 1:100000 dilution. This further strengthen that porin is highly immunogenic.

3.2 Discussion:

We attempted to purify the porin of *S.dysenteriae*type 1 by a method similar to those described for other bacterial porins. Isolated culture was performed in a selective medium for shigella spp. i.e. XLD medium to obtain the particular organism and TSI slant indicated the presence of Shigella spp. specifically.

XLD contains lactose, sucrose and xylose as the fermentable carbohydrates, and phenol red as pH indicator. Bacteria that ferment none of these sugars, e.g., Shigella appear as red, translucent colonies, while lactose fermenters like Escherichia coli cause a rapid fermentation of lactose resulting in acidic pH that is indicated by the formation of yellow colonies. Since Salmonella ferment xylose as readily as coliforms, a second differential mechanism, lysine decarboxylase, is utilized. Those organisms that ferment xylose as well as decarboxylate lysine exhaust the xylose rapidly and the lysine reaction causes a pH reversal to the alkaline reaction similar to Shigella. Lactose and sucrose are added in excess to prevent the same

reversion by lysine-positive coliforms. Sodium thiosulfate and ferric ammonium citrate are indicators of hydrogen sulphide production only when alkaline condition exists; Salmonella will, therefore form red colonies with black centers in 24 hours while hydrogen sulphide production of Citrobacter and Proteus is greatly delayed. Sodium Deoxycholate is added to inhibit the growth of Gram positive bacteria and to tetrad the growth of many strains of coliforms. Our data show the formation of red translucent colonies of Shigella on the XLD agar plate.

TSI agar is used for the determination of carbohydrate fermentation and hydrogen sulfide production in the identification of gram-negative bacilli. TSI agar contains three sugars(dextrose, lactose and sucrose), phenol red for detecting carbohydrate fermentation and ferrous ammonium sulfate for detection of hydrogen sulfide production (indicated by blackening in the butt of the tube). Carbohydrate fermentation is indicated by the production of gas and a visible colour change (from red to yellow) of the pH indicator, phenol red, while, the production of hydrogen sulfide is indicated by the presence of a precipitate that blackens the medium in the butt of the tube. To facilitate the detection of dextrose-fermenting and non-lactose fermenting organisms like Shigella that only ferments dextrose, the dextrose concentration is one tenth the concentration of lactose or sucrose. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidized rapidly, causing the medium remains red or reverts to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension. Formation of yellow butt with a red slant is thus typical of such organisms. Our results that show yellow butt and red slant of TSIA confirm the organism to be Shigella.

Purification of the MOMP to homogeneity was achieved by size exclusion chromatography after selective extraction with detergents. During purification of the SDS-resistant oligomeric form (molecular weight 78000), the peptidoglycan-porin complex was dissociated by performing the extraction with SDS at RT in the presence of 0.4 M NaCl. Moreover, in order to avoid spontaneous aggregation of the native form, the chromatography was performed in the presence of SDS. The MOMP (monomeric form molecular weight 38000) of *S.dysenteriae* type 1 was purified by procedures similar to those used for bacterial porins, and when assessed for its ability to form transmembrane aqueous channels, the MOMP was found to possess pore-forming ability (Roy et al.,1999).

The outer-membrane of gram-negative bacteria is immunologically important structure because of their accessibility to host defense mechanisms. In this study, we have found that antibody to porin was present in the sera of mice immunized with purified porin. Western blot analysis revealed that anti-sera obtained from C57BL/6 mice reacted strongly and specifically with porin of *S.dysenteriae*type 1, implicating the protein (porin) to be an antigen.

Recognition of porin of *S.dysenteriae* type 1 in a whole-cell ELISA by antibody raised against MOMP suggested that *S.dysenteriae* type 1 MOMP is surface exposed, as is known for porins of other bacteria. The surface exposure of porin makes it easily accessible to the host immune system, a criteria that is necessary for any potential vaccine component. Besides being strongly immunogenic and exposed to the surface, the MOMP of *S.dysenteriae* type 1

was found to be antigenically related to those of other Shigella spp. This was proven unanimously as antisera of mice injected with porin of *S.dysenteriae* could recognize porin of both *S.dysenteriae* and *S.flexneri*. The antigenic relatedness of porin would make it useful as a potential immunogen-crossing barrier of species specificities. This cross-reactivity of porin satisfies the criterion of multivalency of Shigella vaccine, which would enable it to be effective against all the different species, as presence of the different strains and serogroups of Shigella raises the demand for a multivalent vaccine that would represent the prevalent species and serotypes.

4. Acknowledgement:

The whole work was performed in the Division of Immunology, National Institute of Cholera and Enteric Diseases, Kolkata – 700010 under the supervision of Dr. Tapas Biswas, Scientist F. I am very grateful to Dr. Tapas Biswas for his advice and guidance.

References

1. Benz, R., A. Schmid, and R. E. W. Hancock.. Ion selectivity of gram-negative bacterial porins. *J. Bacteriol.* 1985, 162:722 – 727.
2. Bernardini, M. L., M. G. Sanna, A. Fontaine, and P.J. Sansonetti.. OmpC is Involved in invasion of epithelial cells by Shigella flexneri. *Infect. Immun,* 1993, 61: 3625 – 3635.
3. Clerc, P., A. Ryter, J. Mounier, and P. J. Sansonetti. 1987. Plasmid-mediated early killing of eukaryotic cells by Shigella flexneri as studied by infection of J774 macrophages. *Infect. Immun.* 55: 521 – 527.
4. Cowan, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Pauptit, J. N. Jansonius, and J. P. Rosenbusch.. Crystal structures explain functional properties of two E.coliporins. *Nature* (London), 1992, 358:727 – 733.
5. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 1956,28: 350 – 356.
6. Dutta, S., P. Dutta, S. Matsushita, S. K. Bhattacharya, and S. Yoshida..*Shigella dysenteriae*serotype 1, Kolkata, India. *Emerg. Infect. Dis,* 2003, 9:1471 – 1474.
7. Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.. Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium lauryl sarcosinate. *J. Bacteriol.*1973, 115: 717 – 722.
8. Hale, T. L. 1991. Genetic basis of virulence in Shigella species.*Microbiol. Rev.* 55: 206 – 224.
9. Roy, S., A. B. Das and T. Biswas.. Antigenicity and antigenic relatedness of the outer membrane proteins of Shigella species. *Microbios,* 1994, 79: 55 – 64.
10. Shiga, K.. Sekiribyogenkenkyuhokokudai-chi (first report on etiologic research on dysentery).SaikingakuZasshi, 1897, 25:790 – 810.

Chapter 15

Present Status of Tidal Variations in the Sundarbans Estuary, India

***Jayanta Sutradhar[1] and Debabrata Mukherjee[2]**
[1]Institute of Ecotoxicology and Environmental Sciences, Kolkata-700097, India
[2]Institute of Ecotoxicology and Environmental Sciences, Purulia Chapter, West Bengal, India
***Corresponding author:** jayantasutradhar23@gmail.com

Abstract

Situated in the eastern coastal state of West Bengal, the Sundarbans Estuarine System(SES) is India's largest monsoonal, macro-tidal delta-front estuarine system. It comprises theSouthernmost part of the Indian portion of the Ganga-Brahmaputra delta bordering the Bay of Bengal. The Sundarbans Estuarine Programme (SEP), conducted during 18–21 March 2011 (the Equinoctial Spring Phase), was the first comprehensive observational programme undertaken for the systematic monitoring of the tides within the SES. The 30 observation stations, spread over More than 3600 sq. km covered the seven inner estuaries of the SES (the Saptamukhi, Thakuran, Matla, Bidya, Gomdi, Harinbhanga, and Raimangal) and represented a wide range of estuarine and environmental conditions. At all stations, tidal water levels (every 15 minutes), salinity, water and air temperatures (hourly) were measured over the six tidal cycles. We report the observed spatio-temporal variations of the tidal water level. The predominantly semi-diurnal Tides were observed to amplify northwards along each estuary, with the highest amplification Observed at Canning, situated about 98 km north of the sea face on the Matla. The first definite sign of decay of the tide was observed only at Sahebkhali on the Raimangal, 108 km north of the sea face. The degree and rates of amplification of the tide over the various estuarine stretchesWere not uniform and followed a complex pattern. A least-squares harmonic analysis of the data Performed with eight constituent bands showed that the amplitude of the semi-diurnal band wasAn order of magnitude higher than that of the other bands and it doubled from mouth to head.The diurnal band showed no such amplification, but the amplitude of the 6-hourly and 4-hourly bands increased head ward by a factor of over 4. Tide curves for several stations displayed atendency for the formation of double peaks at both High Water (HW) and Low Water (LW). One reason for these double-peaks was the HW/LW Stands of the Tide observed at these stations. During a stand, the water level changes imperceptibly around high tide and low tide. The existence of a stand at most locations is a key new finding of the SEP. We present an objective criterion for identifying if a stand occurs at a station and show that the water level changed imperceptibly over durations ranging from 30 minutes to 2 hours during the tidal stands in the SES. The tidal duration asymmetry observed at all stations was modified by the stand. Flow dominant asymmetry was observed at most locations, with ebb-dominant asymmetry being observed at a few locations over some tidal cycles. The tidal asymmetry and stand have

implications for human activity in the Sundarbans. The longer persistence of the high water level around high tide implies that a storm surge is more likely to coincide with the high tide, leading to a greater chance of destruction. Since the stands are associated with an amplification of the 4-hourly and 6-hourly constituents, storm surges that have a similar period are also likely to amplify more during their passage through the SES.

1. Introduction:

The largest delta in the world, formed by the distributaries of the rivers Ganga and Brahmaputra (Seidensticker and Hai 1983; UNEP WCMC (1987, updated 2011); Papa *et al.* 2010), is shared by Bangladesh and India. On its west, the delta is bordered by R.Hoogly and on its east by R. Meghna. The Bay of Bengal forms the southern boundary of the delta, which includes in its southern fringes the dense natural mangrove forests, the Sundarbans. The Indian part of the Sundarbans delta is about 40% of the total area. In this paper, we refer to this region lying between 21.25O–22.5ON and 88.25O–89.5O8 E as the Sundarbans Estuarine System (hereafter called SES).

2. The Sundarbans Estuarine Programme (SEP):

Following the precedent set in the Mandovi-Zuari estuarine system, it was decided to conduct the SEP during an Equinoctial Spring Phase of the lunar cycle in the dry period. Logistics and the time required for planning led to the 3-day observations being carried out during 18–21 March 2011. In this section, we present the rationale for choosing the observation stations and describe the methods used.

2.1 Selection of observation 1 stations:

Eventually, 30 stations were selected based on the above criteria: the stations had to cover the entire area of the SEP, jetties had to be available in the neighborhood, and the location had to be suitable for erecting the VTS. 14 of these stations were in Sector 1 (21.625O13 –22.022ON, 88.250O–88.625O14 E) which covered an area of approximately 1814 sq. km. The stations in this sector lay on the Saptamukhi, Thakuran, and the southern part of the Matla.

The remaining 16 stations were in Sector 2 (22.022O–22.375ON, 88.625O–89.017O16 E) which covered an area of approximately 1800 sq. km. The stations in this sector lay on the northern part of the Matla and on the Bidya, Gomdi, Harinbhanga, and Raimangal. Since theSoutheastern part of the SES could not be surveyed, the southernmost (near the mouth) station was Indrapur on the Jagaddal, a distributary of the Saptamukhi, in Sector 1, and the northernmost station was Dhamakhali at the confluence of the rivers Tushkhali and Bermajur in Sector 2.

The mouth of the Matla being the southernmost, its latitude (21.6025O22 N) has been taken to represent the seaface, from which the distance, along the channel, of all the 30 observation stations have been calculated. For each estuary, we use a code, which is simply the first alphabet of its name, to facilitate identification of the stations with the estuaries on which they are situated. The stations have been named according to the estuary on which they are situated. For example, S30 indicates Shibganj, with the “S” signifying its location on the Saptamukhi. Two stations were located at the confluence of two estuaries and their names therefore include the code for both estuaries. These were Raidighi (ST13), which is located on both 1 Saptamukhi and Thakuran, and Melmelia (GH21), which is located on both Gomdi and Harinbhanga. A potential source of confusion is the tradition in the Sundarbans of giving

different names to the same west–east (south–north) flowing channel after its confluence with a south–north (west–east) flowing channel. An example is given by the west–east flowing channels the Barchara– Kalchara–Nukchara–Pukchara; these names are used, respectively, for the channels to the east of the Saptamukhi West Gulley (SWG), between the SWG and the Saptamukhi East Gulley (SEG), and east of the SEG. In such cases, we have used a hyphen, as indicated above, to separate the various names that the channel takes.

2.2 Tides in the Sundarbans Estuarine System:

Altogether, six low and high tides occurred during the 72-hour observation period. When observations started at 4 AM on 18 March, the first Low Water (LW0) had already passed and the majority of the stations failed to observe it. A few of the extreme northern and northeastern stations could, however, capture LW0, which occurred between 4 and 5 AM at these stations. Likewise, LW6, which occurred between 5.15 and 5.30 AM on 21 March, could be observed only at some of the southern stations. The dominantly semi-diurnal nature of the tides in each of the seven estuaries of the SES is captured distinctly in the observed water level variations. The range of the tide as a function of the along-channel distance from the seaface. Tidal ranges at any location are defined as the differences between successive HW and LW levels (HW level minus succeeding LW level) for any tidal cycle and is equal to twice the tidal-wave amplitude at that point. For stations where gaps in the data coincided with the occurrences of HW/LW, tidal ranges were computed using the corresponding predicted values from the harmonic analyses of the water-level time series (Section 4), provided the phases were found to match. The general features were as follows. As expected for the Equinoctial Spring Phase, HW levels increased over successive tidal cycles at individual stations. The tidal range was significantly lower during the first tidal cycle at several locations. This difference between the first two cycles was higher on the Matla and Bidya. The largest change in range from the first cycle to the second occurred at B16 (Jharkhali Bali). The range increased from mouth to head in all estuaries. On the Saptamukhi, Matla, and the surveyed part of the Harinbhanga, the range increased till the last station. On the Thakuran, Bidya, and Raimangal, the range decreased at the last station, and this decrease was also seen over the last two stations on the Gomdi, which was the narrowest and shortest of the channels surveyed.

2.3 The stand of the tide:

Since the tidal water level curve is sinusoidal, the rate of change of water level (WL) is low around high and low tide. At some places, however, the water level has been noted to remain practically stationary during HW or LW, the change in the level being so slow as to be imperceptible. Such a state has been called the "Stand of the Tide "or "Platform Tide" (NTC 2010; NOAA 2000). This phenomenon has been ascribed to purely tidal causes and is not connected to slack water or tidal currents. A tidal stand is known to prevail in some parts of the world that have complex tidal regimes consisting of double HW/LWs or HW/LW stands.

3. Summary and discussion:

The observed variations of tidal water levels at 30 locations situated on various estuaries of the Sundarbans Estuarine System (SES) have been presented and discussed in this paper. It is for the first time that such data, consisting of continuous measurements of tidal elevations at 15-minute intervals over a 72-hour period, have been reported from this region. The observation stations,

located on all the principal estuaries within the SES covered a wide range of conditions such as estuarine cross-sections, meanders, depths, and confluences with west–east channels. The dominant semi-diurnal tide propagated to all parts of the SES, with an overall northward amplification. The first definite sign of decay was observed only at station R29 (Sahebkhali) on the Raimangal. The lowest mean tidal range (4.32 m) among all 30 stations was observed at S2 (Dhanchi), while the highest (6.73 m) occurred at M26 (Canning). The pattern of amplification was, nevertheless, complex because the SES consists of not only the south–north-flowing main channels, but also fairly big west–east- oriented channels connecting them. For example, the low tidalranges and HW levels observed at S2 are possibly due to the interactions between the tidal wave propagating north up the Jagaddal and the westerly tidal inflow from the Thakuran through the Dhanchi Khaal. Likewise, the tidal range was also low at M4 (Bonnie Camp), which too was located in a connecting channel. In general, however, the tidal range increased from mouth to head, that is, the tidal wave amplified northwards. A simple explanation for this amplification can be given as follows. Channel geometry and frictional dissipation are the most important factors that determine the amplification or decay of a tidal wave as it progresses head wards along an estuarine channel. All the major estuaries in the SES are funnel-shaped; with widths decreasing rapidly head wards (northwards) from their mouths (Section 1), i.e., they are convergent channels. This type of estuarine geometry has a 'funneling effect', which tends to amplify the tide as it propagates towards the head of the channel. Frictional dissipation on the other hand, tends to decrease this amplification. The northward amplification of the semi diurnal tide observed in general in the SES channels therefore implies that, on an average, the geometric effect dominates over frictional dissipation in the SES.

4. Acknowledgements:

This work was conducted as part of the research project at SOS, JU (Sanction No. F/INCOIS/INDOMOD-11-2007/2691 dated December 20, 2007) funded by INCOIS, Ministry of Earth Sciences, Government of India, under the INDOMOD programme. The authors gratefully acknowledge the following organizations and people whose encouragement and active cooperation led to the successful implementation of the Sundarbans Estuary Programme: The Indian National Centre for Ocean Information Services (INCOIS), Ministry of Earth Sciences, Government of India, for funding the Programme; the Department of Forests and the Sundarbans Development Board, Government of West Bengal for permission to work in some of the Reserve Forest areas, accommodation and other facilities; the Tagore Society for Rural Development (TSRD), Sabuj Sangha (SS), and the Calcutta Wildlife Society (CWS) for providing local manpower and logistic support; the Border Security Force (Eastern Frontier), Government of India, and the West Bengal Police for providing security during the observation period.

References

1. Tidal Variations in the Sundarbans Estuarine System, India. M.Chatterjee et al. Journal of earth system science.
2. http://www.shrimperowners.org/sitefiles/Poole%20Tides.pdf
3. http://iwai.gov.in/piwtt.htm
4. http://www.salinesystems.org/content/6/1/8
5. http://www.ecology.uni-corvinus.hu
6. http://www.bom.gov.au/oceanography/projects/ntc/ntc.shtml etc.

Chapter 16

Biodiesel from Algae: A Potential from Sundarban

***Shreyasee Roy[1], Mayukh Barik[2], Debabrata Mukherjee[3], Gourab Choudhury[4], Jyotiskona Barik[3]**
[1]Department of Botany, Vidyasagar University, Midnapur, West Bengal, India
[2]Department of Environmental Science, Vidyasagar University, Midnapur, West Bengal, India
[3]Institute of Ecotoxicology and Environmental Science, Kolkata, West Bengal, India
[4]Department of Environmental Management, Kalyani University, Kalyani, West Bengal, India
***Corresponding author:** roy.shreyasee@gmail.com

Abstract

Fuel is one of the primary necessities in these days for humans to run their households to industries. It is also vital for the transportation. The fossil fuel is the main source of supply. But a number of environmental as well as economical problems are associated with the use of fossil fuels. The combustion of fossil fuels causes serious environmental hazards. The amount of fossil fuel is also limited and is not equally distributed all over the world. After many analyses to sustain world's resources, fossil fuels are partially replaced by biofuel. Biodiesel also has received a considerable attention in recent years as several companies are likely attempting to commercialize microalgal biodiesel after the technology for production and use of biodiesel is introduced. Algae can be the most efficient biological producer of biodiesel because of their higher photosynthetic efficiency, higher biomass productivity, faster growth rates, higher CO2 fixation and O2 productivity, growth ability in variable climatic conditions or in non-agricultural lands or in non-potable water and less water requirements. So algae can be considered as a source of sustainable green energy. This paper leads with potential of biodiesel production from algal species particularly found in Sundarban.

Key Words

Algae, Biodiesel, Sundarban, Sustainable, Green Energy

1. Introduction:

Fuel represents almost 70% of the global energy used for transportation, manufacturing anddomestic heating and these are mainly fossil fuels (Gouveia & Oliveira, 2009). The simpletechnology of extraction of oil has increased the use of these fossil fuels. However, several environmental as well as economical problems are associated with the use of fossil fuels. The combustion of fossil fuel results in the production of greenhouse gases and other pollutants like unburnt hydrocarbons, carbon-monoxide (CO) and nitrogen oxides (NO and NO2). In comparison to carbon dioxide, nitrous oxide is 200 times more efficient in infrared absorption. Thus, to sustain world's resources the use of biodiesel has **came in recent concerns.**

To displace the petroleum derived transport fuels, biodiesel also has received a considerable attention in recent years after the introduction of the technology of production and use of biodiesel (Nigam & Singh, 2010). The concept of biodiesel was likely introduced in 1895 when Rudolf Diesel designed an engine to run on peanut oil (Singh & Singh, 2010). Biodiesel is generally derived from vegetable oils or animal fats. First-generation biodiesel were derived from crops such as sugar cane, corn and soybean that results to water scarcity and deforestation. Second-generation biodiesel were originated from lignocelluloses and forest residues which need large areas of lands that could be used for food production. Biodiesel derived from algal feedstock is the third-generation biodiesel based on technology projections. Algae are considered to be an alternative energy source without the drawbacks of the first- and second-generation biodiesels. The most important aspect for using algae as renewable source is their rapid growth rate and high lipid contents. Compared to food crops algal growth rates are 20-30 times faster. The algal feedstock does not come into the competition with food crops for land use as harvesting can be done in uninhabited, from salt-water marshland to deserts. Thus also demands no fresh water supply.The Sundarban delta is a link between the terrestrial and estuarine aquatic ecosystem. A large number of plant species diversity is observed in this deltaic region and algae is one of the dominating species. Thus, to open up the potential of algal species from the Sundarban delta for producing biodiesel this study has been done in all over the Sundarban delta (Fig. 1) and was very particular on three species-*Enteromorpha intestinalis, Ulva lactuca* and *Cattenella repens.* This paper highlights on the potentiality of algal species from Sundarban based on their growth rates and fat contents.

2. Study sites

The lower Ganga delta of the Sundarban was formed by the step by step deposition of the fine clay, silt and sand particles accelerated by the tidal thrust of the sea. At the mouth of the river systems the "Swatch of No Ground" causes continuous silt sedimentation upon the river banks. Thus, the Sundarban belt was developed. The total geographical area of the Indian part of Sundarban is 9630 km2, within the latitude of 21°31'N to 22°30'N and the longitude of 88°10'E to 89°51'E with an estimated average annual rainfall of 1500-2000mm, average humidity of 60-90% and temperature variations within 12°C-35°C (Naskar and Mandal,1999).

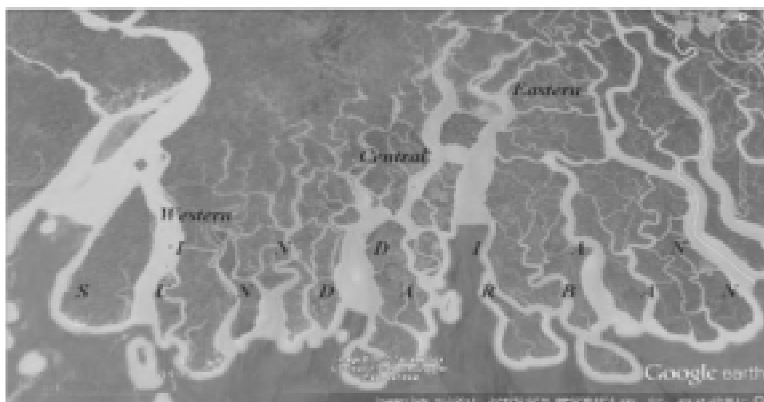


Fig.1: The all over Sundarban delta

3. Materials and Methods:

Algal strains were collected from different aquatic fields and studied under the microscope for identifications. The species were separately inoculated in certain amounts and the initial weights were recorded. Different types of culture systems were followed. *Enteromorpha intestinalis* is cultured by net culture technique (Fig.2 B). For *Ulva lactuca*two separate culture systems were practiced to find out the highest production. This species was cultured at a time on substratum and by floating raft culture technique separately. Single raft culture technique was followed for *Cattenella repens*(Fig. 2A). After regular intervals final weights were also recorded. The daily growth rates and growth rate percentages (Graph 1) were calculated from these data for all species. These methods were repeated in different seasons of a year. At the time of the final harvesting the biomass were calculated and fat contents (Graph 2) were measured by following the standard methodology of APHA after 1995.

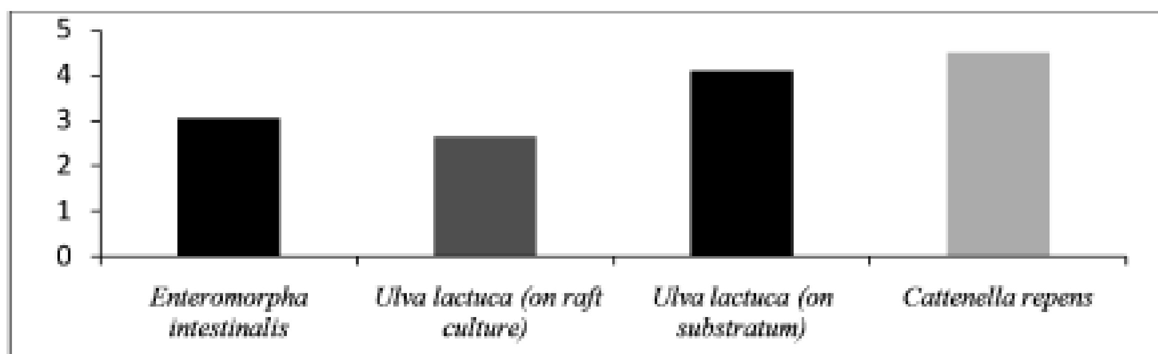
(A) (B)

Fig. 2: (A) Single Raft Culture of *Cattenella repens,*
(B)Net culture of *Enteromorpha intestinalis*

4. Result and Discussions:

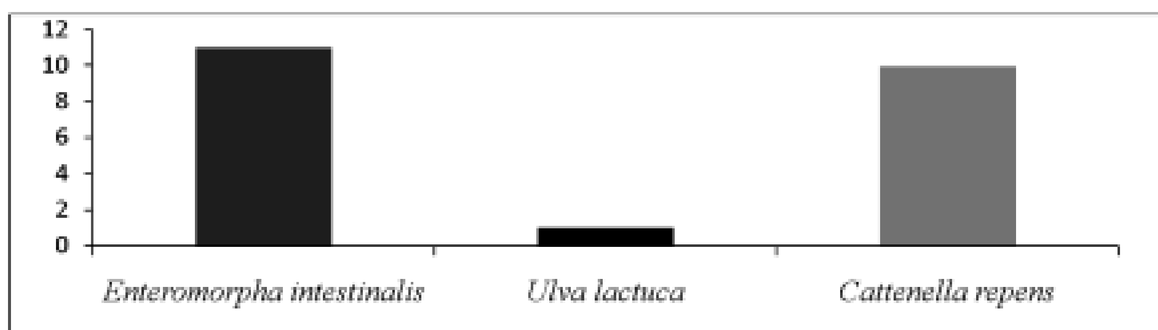
In the Sundarban delta the dominating algal species are- *Enteromorpha intestinalis, Ulva lactuca* and *Cattenella repens.Cattenella repens* is found in huge amount in all over the Sundarban area. These three species are thus considered as best potential species from this area.

Compared to other species the daily growth rates (DGR) of these species are very high which is a very important point for considering algal species for commercial biodiesel production. For *Cattenella repens* the DGR is found highest (4.5%). *Ulva lactuca* when cultured on the substratum showed a growth rate of 4.09% and in case of floating raft culture the growth rate of *Ulva lactuca* is lowest (2.65%). The same of *Enteromorpha intestinalis*is 3.5%. This result shows that the growth rate of *Cattenella repens* is almost same with *Ulva lactuca* cultured on substratum.



Graph 1: Daily Growth Rate Percentage (DGR%) of the studied algal species

Finally the fat content of these species are calculated by following the standard methodology of APHA after 1995. The highest fat containing species is *Enteromorpha intestinalis*with a fat content of 11%. In *Cattenella repens*the fat content is 10%. *Ulva lactuca* has only 1% fat content.

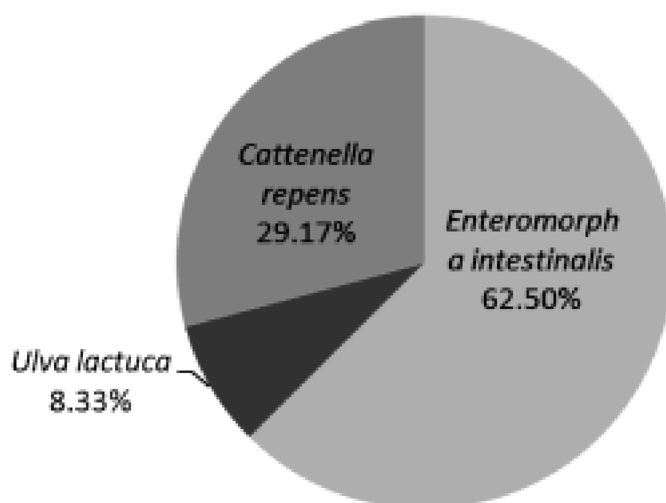


Graph 2: Fat (%) of the studied algal species

5. Conclusion:

The daily growth rate of *Cattenella repens* is almost 10% greater than *Enteromorpha intestinalis*. But on comparison on fat content the probable oil productivity is highest in *Enteromorpha*. For *Cattenella repens* fat content is also higher. Thus it shows that *Cattenella repens* has probably the highest potentiality to be a good renewable source of biodiesel whereas due to very low oil content use of *Ulvalactuca* as a renewable source may not be advisable because for commercially viable biodiesel faster growth and high fat content both are important.

From an area of 11-12 sq. mtr of culture of *Enteromorpha intestinalis*, after one month the total biomass productivity is 1.5-2.5kgs where as for *Cattenella repens*that is 700-750gms. The total production of *Ulva lactuca* is always very low and found 200-250gms. So it can be concluded that from an area of 11-12sq mtr a one month culture of *Enteromorpha* has the highest oil productivity (62.50%), 29.17% from *Cattenella* and 8.33% from *Ulva.*



Graph 3: Percentage potential (%) of biodiesel productivity from unit area

However, the production of biodiesel from algae is commercially viable only if the production cost is lower than the traditional fuel. For culture and harvesting of algal species, use of open culture system is most suitable as natural light and CO2 supply is continuous.

Abbreviations:

APHA	American Public Health Association
DGR	Daily Growth Rate
SRC	Single Raft Culture

References

1. Gouveia L. and Oliveira A.C. Microalgae as a raw material for biofuel production. J. Ind. Microbiol. Biotechnol, 2009, 36,269-274.
2. Naskar, K.R. and R.N. Mandal. Ecology and Biodiversity of Indian Mangroves. DayaPublishing House, New Delhi, India.,1999, 754p.
3. Nigam P. and Singh A. Production of liquid biofuels from renewable resources. Prog. Energy Combust. Sci., 2010
4. Singh S.P. and Singh D. Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: a review. Renewable and Sustainable Energy Reviews.,2010, 14,200-216.

Chapter 17

Possible bio-mining by metal tolerant bacteria

Rajanya Bhattacharyya, Monica Ghosh, MoupiyaGiri, AnkitaSen, *Debjani Dutta
P.G.Department of Microbiology, St.Xavier's College, Kolkata – 700 016.
***Corresponding author:** dunaliella11@gmail.com

Abstract

In this investigation, effluents in the form of solid slags were obtained from the Titagarh Steel Melting Workshop having a pH around 7.5 and electrical conductance 303.4S/m. The high electrical conductance indicates the presence of impurities in the form of metals in the effluent. Three different bacterial strains (C-I, C-II and C-III) were isolated from it, two of which were Gram negative and a third one Gram positive in nature. Biochemical tests performed with each of the three different organism inferred C-I and C-II showing catalase activity whereas C-I and C-III showing oxidase activity. They were then assessed for their possible role in the detoxification of industrial waste. Chromium appears in the discharged effluent of iron and steel industries as they are used in steel processing. Experiments were designed so as to determine the chromium (VI) tolerating capacity of these strains. The three strains were found to tolerate chromium (VI) up to a concentration of 30 – 40 g/L. These bacteria were also checked for their ability to take up iron. Growth was observed upto an iron concentration of 30 ppm. The iron uptake ability was further confirmed by atomic absorbance studies. A decrease in the concentration of iron in the broth was observed due to growth of organisms. The C-II appearing as Gram +ve rods was maximum efficient in removing Cr and Fe from the medium. Upto 45% chromium removal and growth till 30ppm concentration of iron was exhibited by the latter. 16srRNA analysis identified this organism as ***Bacillus cereus strain IHB B 379*** (GenBank Accession Number: **KF475795.1**). TEM studies done with C-II strain showed maximum tolerance to chromium, confirming the deposition of chromium particles within the cells. These findings have profound implications as these organisms can be utilized in cleaning up industrial and other wastes containing high concentrations of toxic metals and thus prevent further pollution of the environment.

Keywords:

Chromium (VI), iron (Fe), metal tolerance, catalase, oxidase

1. Introduction:

Rapid industrialization and increasing urbanization, discharging of heavy metal effluent have resulted in excessive contamination of the environment. This environmental pollution is posing a great threat not only to mankind but to the entire living world. Therefore increasing

awareness has been growing rapidly worldwide to abate this nuisance. Various effective techniques have been employed to treat industrial effluents before discharging them. Recently, biological organisms are being utilized in a process called bioremediation.

We have gone about the project keeping this phenomenon of bioremediation in mind and evaluating if the bacterial species isolated from the steel effluent possesses any such potential or not. Since Iron and steel industries involve the use of chromium in iron welding process, these bacterial isolates must be tolerant to chromium and iron stress to a certain degree. Under laboratory conditions they did show results that assure us their capability of enduring and utilizing metals in for growth.

2. Materials and Methods:

2.1 Physical and chemical characterization of solid waste from steel industry:

The sample was examined physically for its shape, size, colour, texture and odour. Chemical tests on pH, metal test by Flame testing, electrical conductance (at 30°C) and solubility was checked.

2.2 Isolation of microorganisms from the steel effluent:

Using Pour plate technique, solid slag suspension in sterile water as mixed with autoclaved molten nutrient agar (Peptone-5gm NaCl- 5g, Beef extract- 3g, Distilled water-1000ml at pH-6.9) aseptically and incubated at 37°C overnight. Further pure cultures were obtained.

2.3 Gram staining of the pure cultures obtained:

Gram character of the isolated pure culture of microorganisms was carried out by staining.

Heat fixed smears of microorganisms were flooded with Crystal violet for 1min and washed. Gram's Iodine and alcohol were added next followed by the counterstain- Safranin. The air dried slide was then examined under a Compound microscope.

2.4 Biochemical tests:

Further identification of the isolated bacterial strains was carried out by the Catalase and Oxidase test.

1) Catalase test- The pure bacterial cultures were smeared on grease free slides followed by 3% H_2O_2. The slides were observed for effervescence. If there is any effervescence, then the corresponding culture is catalase positive and vice versa.
2) Oxidase test- N,N,N,N'-tetra methyl Paraphenylenediamine (TMPD) was added dropwise on Whatman filter papers inoculated with our pure cultures. The area of inoculation turning to dark blue to almost black indicates a positive oxidase activity by the bacterial strain concerned.

2.5 Evaluation of Chromium tolerance:

10g of $K_2Cr_2O_7$ was added to 100ml of sterile water to prepare a stock solution of concentration of 100g/l. Nutrient broth having different volumes of chromium solution was made from the mother stock. The different concentrations were 10,20,30,40 and 50g/l. The tubes were inoculated with log phase cultures and kept for incubation at 37°C for 24hours.

2.6 Transmission Electron Microscopy (TEM) studies:

TEM studies were performed in collaboration with Indian Association for the Cultivation of Science, Kolkata.

2.7 Evaluation of Iron tolerance:

Nutrient broth containing different concentrations of iron solution (10, 20, 30, 40 and 50ppm) were prepared. 100ul of log phase cultures were added to each tube of different concentrations and incubated at 37°C for 24 and 48 hours respectively.

Amount of iron left was obtained spectrophotometrically after addition of and phosphoric acid

2.8 Atomic Absorbance studies:

Samples were sent to SociétéGénérale de Surveillance (SGS) India,Kolkatafor atomic absorbance studies.

3. Results:

3.1 Physical and chemical characterization of solid waste from steel industry:

The slag obtained from the steel melting factory was odourless, irregular, slate black colour with reddish, greyish and brownish specks.

The pH was around 7.599 with electric conductance of 303.4S/m and was completely insoluble in acid, base or water.

3.2 Gram character determination of microorganisms isolated from steel effluent:

Gram staining done with three different microorganisms showed that one of them was gram negative, short rod while the rest two were gram positive rods.

Microorganism	Appearance Of Cells Under Microscope	Gram Characteristic
Culture – I	Red coloured; short rods	Gram negative
Culture – II	Red coloured; rods	Gram positive
Culture – III	Purple coloured; rods	Gram positive

3.3 Biochemical tests for identification of isolated microorganisms:

Of the three different bacteria, one showed both catalase and oxidase activity, the second one had catalase activity but no oxidase activity and the third one was catalase negative but oxidase positive.

3.4 Evaluation of chromium tolerance:

All the three different cultures were able to grow even at very high levels of chromium concentrations (30-40g/l). TEM studies done on one of the bacterial cultures (C-II) showed maximum tolerance to chromium, which confirmed the deposition of chromium particles within the cells.

Hence, it can be concluded that all the three cultures are tolerant to high chromium concentrations.

Table:1a- Culture I:

Concentration of Chromium Solution (g/L)	**OD_{520}**	
	0(hr)	**24(hrs)**
Blank (NB)	0	0
10	0.47	0.12
20	0.54	0.14
30	0.66	0.38
40	0.68	0.18
50	0.72	0.23
Positive control (NB + culture)	0	0.05

Table:1b

Initial concentration of chromium in solution(g/L)	**Residual concentration of chromium after 24 hrs (g/L)**	**Chromium uptake by Bacteria (g/L)**
10	5.5	4.5
20	6.5	13.5
30	17.5	12.5
40	8.5	31.5
50	10.5	39.5

Table: 2a- Culture II:

Concentration of Chromium Solution(g/L)	**OD_{540nm}**	
	0(hr)	**24(hrs)**
Blank (NB)	0	0
10	0.43	0.10
20	0.48	0.20
30	0.53	0.17
40	0.59	0.20
50	0.59	0.18
Positive control (NB + culture)	0	0.01

 ISBN: 978-81-928924-3-6

Table: 2b

Initial concentration of chromium in solution(g/L)	Residual concentration of chromium after 24 hrs (g/L)	Chromium uptake by Bacteria (g/L)
10	5.5	4.5
20	10.5	9.5
30	9.5	20.5
40	10.5	29.5
50	9.5	40.5

Table 3a- Culture III:

Concentration of Chromium Solution(g/L)	OD_{540nm}	
	0(hr)	24(hrs)
Blank (NB)	0	0
10	0.34	0.11
20	0.45	0.14
30	0.51	0.15
40	0.53	0.15
50	0.55	0.16
Positive control (NB + culture)	0	0.05

Table 3b-

Initial concentration of chromium in solution(g/L)	Residual concentration of chromium after 24 hrs (g/L)	Chromium uptake by Bacteria(g/L)
10	6.5	3.5
20	8.5	11.5
30	9	21
40	9	31
50	9.5	40.5

3.5 Evaluation of iron tolerance:-

The growth of all the three different cultures was found to be facilitated upto iron concentration of 30ppm. With still higher concentrations of iron, the test tube appeared to be less turbid showing negligible growth.

The iron uptake ability was further confirmed by Atomic absorbance studies.

Table 4: For 24 hours

Concentration of iron in solution (ppm)	Culture I	Culture II	Culture III
-ve control	0.00	0.00	0.00
+ve control	0.37	0.07	0.18
10	0.42	0.11	0.27
20	0.45	0.05	0.22
30	0.32	0.05	0.14
40	0.18	0.12	0.08
50	0.17	0.17	0.13

Table5: For 48 hours-

Concentration of iron in solution (ppm)	Culture I	Culture II	Culture III
-ve control	0.00	0.00	0.00
+ve control	0.34	0.10	0.23
10	0.67	0.10	0.30
20	0.45	0.09	0.28
30	0.23	0.05	0.13
40	0.18	0.12	0.12
50	0.19	0.09	0.20

4. Discussion:

Chromium and iron appears in the discharged effluent of iron and steel industries as they are used in steel processing. Three different bacterial strains that were isolated were then assessed for their possible role in the detoxification of industrial waste.

From the results, it can be inferred that all the three strains could tolerate chromium (VI) up to a concentration of 30 – 40 g/L. 16srRNA studies were done on one of the bacterial cultures (C-II) identified to be as ***Bacillus cereus strain IHB B 379*** (GenBank Accession Number: **KF475795.1)** that showed maximum tolerance to chromium.

These bacteria were then further checked for their ability to take up iron. Growth was observed upto an iron concentration of 30ppm (table 4 & 5). The iron uptake ability was

further confirmed by Atomic absorbance studies. A decrease in the concentration of iron in the broth was observed due to growth of organisms. However, the microorganisms were unable to convert Fe^{2+} to Fe^{3+}, as confirmed by red colouration on addition of acid, but no colour change in its absence.

Disposal of industrial waste and heavy metal contamination are among the major problems of today. Hence, these organisms that have been found to be tolerant to heavy metal stress and also capable of uptake of such metals, can be utilized in cleaning up industrial and other wastes containing high concentrations of toxic metals and thus prevent further pollution of the environment.

References:

1. Overcoming the Heme Paradox: Heme Toxicity and Tolerance in Bacterial Pathogens-Laura L. Anzaldi and Eric P. Skaar
2. Efficient removal of hexavalent chromium by a tolerant *Streptomyces sp*. affected by the toxic effect of metal exposure - Morales - 2007 - Journal of Applied Microbiology - Wiley Online Library
3. Unbound MEDLINE: Chromium tolerance and reduction potential of a *Bacillus sp*.isolated from metal contaminated wastewater.
4. Evaluation of biosorption potency of *Acinetobacter sp*. for removal of hexavalent chromium from tannery effluent - Springer
5. Hydrogenases in sulfate-reducing bacteria function as chromium reductase - Springer
6. Ferric iron reduction by sulfur- and iron-oxidizing bacteria.
7. Biology of Iron-and Manganese-Depositing Bacteria - Annual Review of Microbiology, 38(1):515

Chapter 18

Eco-tourism: A Scpoe for Generation of Livlihood & Environmental Management in District of Purulia, West Bengal, India

***Debabrata Mukherjee[1], Mrinmoy De[2], Jyotiskona Barik[1], Shreyasee Roy[3], Gourab Choudury[4], Mayukh Barik[1], Aditi Bose[1]**

[1]Institute of Ecotoxicolgy & Environmental Science, Kolkata, West Bengal, India

[2]Ranaghat Bharati High School, Kamarpara, Ranaghat, W.B., India

[3]Department of Botany, Vidyasagar University, W.B., India

[4]Department of Environmental Management, University of Kalyani, W.B., India

***Corresponding author**: debprl@yahoo.co.in

Abstract

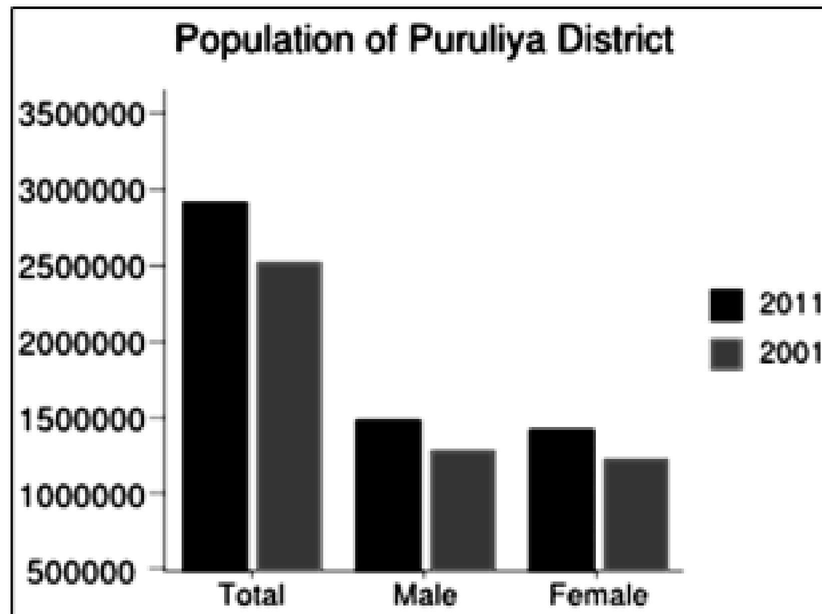
Ecotourism is defined as "responsible travel to natural areas that conserves the environment communities around the world to fight against poverty and to achieve sustainable development" (http://ecotourismbengal.wordpress.com). With an emphasis on enriching personal experiences and environmental awareness through interpretation, ecotourism promotes greater understanding and appreciation for nature, local society, and culture. The district of Purulia in West Bengal, India has its own importance politically, culturally as well as ecologically. The nature of this particular region has numerous potential to flourish the eco-tourism industry in the state. Additionally, the local inhabitants will be benefitted through the eco-tourism since it will generate livelihood for the people and revenue for the state. Besides these, the geographical, topological importance of this zone can be maintained through the environmental management provided by the process of eco-tourism in the district.

1. Introduction:

Purulia came into being as a district of West Bengal in 1956. Purulia is the westernmost district of West Bengal with all-India significance because of its tropical location, its shape as well as function like a funnel. It funnels not only the tropical monsoon current from the Bay to the subtropical parts of north-west India, but also acts as a gateway between the developed industrial belts of West Bengal and the hinterlands in Orissa, Jharkhand, Madhya Pradesh and Uttar Pradesh.

This district is between 22°42'35" and 23°42'0" north latitude and 85°49'25" and 86°54'37" east longitude. Midnapore, Bankura and Burdwan district of West Bengal and Dhanbad, Bokaro, Hazaribagh, Ranchi, West Singbhum, East Singbhum district of Jharkhand State bound this district. Geographical location of this district is very important since it is the border district of the state of W.B. and is used as the corridor for transportation (http://purulia.gov.in/index.htm).

The demographic profile of the district shows clearly that the population is increasing sharply but opportunity of earning is still limited.



Out of the 2468 rural inhabited mouzas of this district 994 mouzas has been declared as backward i.e. 40.28 % of the inhabited mouzas are backward. The people should be provided with alternative sources of earning their livelihood (http://www.census2011.co.in/).

Ecotourism, to be successful, must promote sustainable development by establishing a durable productive base that allows local inhabitants and ecotourism service providers to enjoy rising standards of living. The rich cultural heritage of the district can be a good advantage

 ISBN: 978-81-928924-3-6

to flourish the eco-tourism in this region. This is a place where we can observe the culture of Bengal, Bihar (recent Jharkhand) and Orissa are in harmony. Starting from archaeological evidences to local festivals, every cultural event has got a nice tribal touch in it, which is the specialty of Purulia. The archaeological evidences or the antiquities present in Purulia district makes it a potent place of attraction for the tourists with the extensive forest and forest resources is the good haunting place for the nature lovers.

2. Specific sites for eco-tourism in Purulia:

Places of Attraction –

2.1.1 Ajodhya Pahar:

Ajodhya Pahar is located in the southwest of Purulia. It is about 42 kms. from Purulia town via Sirkabad. It is a famous place of tourism. It is a woody hill having a tableland above. It is about 2133 ft high from sea level. Numerous small streams drain its western and southern slopes into the river Subarnarekha and the northern slopes into Kasai and Kumari River.

2.1.2 Matha:

Matha Buru (Matha hills) is commonly known for its esthetic beauty. Annual 'Mela' is originated on the hill by tribal community. Many nature camp and Rock Climbing courses are conducted by different organization mostly during winter season. Last year number of such camps was 31 and more than 2000 school &college students participated. Local Forest Protection Committee members are engaged for providing catering facilities for camp and some of them also act as local guides.

2.1.3 Pakhi Pahar:

It is another tourist attraction with vicinity along with scenic beauty of Pardi Dam.

2.1.4 Eco-Shop:

An unique attempt to provide facility to the local villagers to sale their handicraft produce to the tourist.

2.1.5Gachbari (Tree house):

First time in South Bengal to provide tourist a feeling of staying above a tree.Under construction at Matha Range shortly to be completed.

2.1.6Dowry Khal:

Originating place of Sobha River, a tributary of Subarnarekha.Known for its natural beauty and a favourite camping place for adventure tourist.

2.1.7Purulia Pump Storage Project:

900 M.W. capacity hydroelectric project. Upper dam surrounded by lash green Sal forest is a potential place for tourist destination.

2.1.8 Tugga and Bramni Falls:

To perennial streams near Bagmundi favourite for day visitors and picnic party in winter.

2.1.9Nature Interpretation Centre at Ajodhya:

Under construction.Aimed to represent Ajodhya Eco-system along with tribal culture and tradition to the visiting tourists. Shortly to be completed.

2.1.10Sirkabad:

Base camp for rock climbers and trekkers who come to help rock climb courses at Gaja Buru, the top peak of Ajodhya Hill.

2.1.11 Dams surrounding Ajodhya Hill:

Murguma, Pardi, Burda, Ramudih, and Gopalpur are few dam sites that are liked by picnic minded people and tourists. They not only represent the scenic beauty but also serves as spot for bird watching.

2.1.12 Panchet Pahar:

This hill is situated in the northeast of Raghunathpur Town. It is 5 km. long and stretched from north to south in a long rounded ridge with a height of about 2110 ft. above the sea level. At the foot of the hill towards the southern end are the ruins of the fort of the powerful Rajas of Panchakotraj.

The Reservoir on the junction of Damodar and Barakar River is in front of this hill. Panchet Hill is also famous for huge reserve of medicinal plants. "Wild Wide Fund for Nature India" has conducted a survey in this regard. The survey reveals that 210 types of medicinal plants are available in Panchet Hill**,** which will be very useful if harnessed properly.

Table.1 Forest Rest-house under Purulia Division:

Location of Forest Rest-house	Number of room available	Charge/ Night	Bed Capacity in Dormitory	Rent of Dormitory	For Booking	Contact Number
1	**2**	**3**	**4**	**5**	**6**	**7**
Ajodhya	2	500/-	-	-	D.F.O. Purulia Division	03252-222329
Balarampur	1	300/-	-	-	- Do -	- Do -
Matha	1	300/-	3	200/-	- Do -	- Do -
Jhalda	1	250/-	-	-	- Do -	- Do -
Purulia	1	500/-	-	-	- Do -	- Do -
Arsha	1	200/-	10	Per head/ day 50/-	- Do -	- Do -

(Sources: Divisional Forest Officer, Purulia Division, Purulia)

3. Importance of eco-tourism in Purulia:

- Purulia is a drought prone district. The district has a sub-tropical climate and is characterized by high evaporation and low precipitation. Temperature is very high in summer and low in winter – it varies from 3.8^{o}C in winter to 52°C in summer, causing dryness in moisture. Average annual rainfall varies between 1100 and 1500 mm. But uneven, scanty and erratic rainfall results agricultural drought in the kharif season. Only ecotourism industry can create some sources of earnings for the local people by least investment and by an easy manner. Demand is mainly due to—-
- Other forms of industry not sustainable

- Warm, friendly and hospitable people
- Competitive advantage
- Local employment
- Economic upliftment
- Local knowledge and pride
- Rich Natural and Cultural Resource base

4. Environmental Impact Assessment:

An important work to be done prior to make a planning of eco-tourism is the EIA of the sites. It involves the following –

- Understanding local factors
- Sustainability parameters
- Criteria for measurement
- Monitoring

To make the planning of eco-tourism physical designing is an important step to be done.

1. Use of local materials- Local materials should get first priority in ecotourism. This zone is the producer of some beautiful earthen and lac materials. Tourists should be provided the facility of using those.
2. Regulation— Local panchayat and general trained people should be involved in the process.
3. Zoning— The district has the places scattered in different position. A clear zoning is to be done to make an ecotourism map.
4. Ecotechnology—It involves conservation of water, soil and energy. Ecological forces should utilize.

5. Recommendation:

Since, the location and the political scenario of Purulia district is getting prime importance day by day nationally as well as internationally due to specific movements that took place recently, to implement eco-tourism in a better way in Purulia some important aspects should be kept in mind.

These are to be:

1. Reducing leakages and improving linkages: Improved linkage among different sectors of Govt. & non-govt. Organizations is needed to maintain a proper balance in the whole process of management.
2. Tourism industry: This should be provided with modern facilities to attract tourists by providing standard resorts, arrangements of amusements, basic facilities of drinking water, electricity, internet etc.
3. Local transportation industry: Not only the govt. Transport facility but also local co-operatives should be encouraged to provide transport taking government aids.
4. Agriculture and fishing: Though this district is drought prone but encouragement should be given to the farmers to involve them in agriculture and fishing by providing them high yielding seeds of crops.
5. Construction sectors: To provide a developed transport system construction of new roads and repairing of the existing roads are needed. Specially, the condition of the

national highways (NH 32) are extremely bad, those are to be repaired in an immediate effect.

6. Handicrafts and souvenirs: Local small scale industries should be cared with utmost intensity to attract tourists at the same time it will be helpful to improve their financial status. Handicrafts associated with lac industries are the best example of this kind.
7. Local art forms: Purulia has its own rich culture and art forms. Many local artists are involved in different cultural art forms like Chou dance, Jhumur, karam dance etc. Regional culture should be cultured and reared to make eco-tourism more effective and successful.

References:

1. http://ecotourismbengal.wordpress.com/you-on-ecotourism-in-bengal/
2. http://ecotourismbengal.wordpress.com/conference-prospects-of-eco-tourism-in-north-bengal-sukna-dec-03-2006/eco-tourism-destination-west-bengal/
3. http://ecotourismbengal.wordpress.com/conference-prospects-of-eco-tourism-in-north-bengal-sukna-dec-03-2006/ecotourism-issues-and-concerns/
4. http://www.mapsofindia.com/maps/westbengal/districts/
5. http://purulia.gov.in/index.html
6. http://www.census2011.co.in/

Chapter 19

Stack Farming: A Step Ahead Towards Food Security

***Gourab Choudhury, Jyotiskona Barik, Debabrata Mukherjee, Mayukh Barik, Shreyasee Roy, Aditi Bose and Mrinmoy De**
Institute of Ecotoxicology and Environmental Science, Kolkata-700097, India
***Corresponding Author:**cemk.gourab@gmail.com

Abstract

With increasing population, agricultural land is reducing rapidly and food crisis is increasing. Time has come to take some strategies to make more space suitable for farming which should be innovative as well as sustainable. Stack farming or vertical farming can potentially be a way out for this. It can be applied for any kind of land like highly saline soil, laterite soil, degraded land etc., and even in the urban regions. This is a process which results proper utilization of resources e.g. water, energy etc. and higher yield of production. Stack farming is a properly managed process that also promotes organic farming, supports a good economy generation for farmers and leads the society towards sustainable development. Present paper deals with an experimental set up done in Aruar (23°27’15.92"N, 87°52’11.00"E), a small village of Burdwan district, West Bengal. Twelve sets of bamboo stacks with two stairs were made. A mixer of rotten straw, compost and soil was spread on each stack. On the base, major crop (potato) was given. Middle stack/ bed was 4 ft. above the ground so that at least defused sunlight can be available for potato (*Solanum tuberosum).* On the middle bed there were Pea (*Pisum sativum*), Spinach (*Spinacia* sp.), Chilli (*Capsicum* sp.), Tomato (*Solanum lycopersicum*), Beans (*Phaseolus* sp.) and Dhania (*Coriandrum sativum*). On the top stack there were Onion (*Allium cepa*), Tomato, Beans and Dhania. The yield was quite satisfactory with multiplied cultivable land in this process.

Keywords

Stack or vertical farming, sustainable and organic farming, alternative livelihood.

1. Introduction

If human can live in high-rise buildings, manipulate their area by increasing it in vertical direction, why not plants. It is predicted that the world population will reach 9 billion by 2050, of which 70% will live in urban centers. This change, alongside a changing climate, will strain Earth’s resources, specifically the ability to supply foods. A valuable investigation would be to determine other ways to supply food to cities alongside current agricultural practices in a sustainable manner (Malek Al-Chalabi, 2010). With this idea, some farmers were motivated to give their time and efforts, so that a model set-up can be tested. One stack was made on a

potato field without hampering the major crop. The set-up and maintenance cost was provided to the farmers; they looked after the set-up properly and whatever yield they could reach, was theirs but no wedge were given. Organic farming was performed on the stacks. It was one tricky attempt to motivate the farmers towards organic farming with the process according to their knowledge of organic farming.

Inglobal scenario, stack farming or vertical farming is hugely practiced for producing fodder, some vegetables and bottom mushrooms but the processes which are used, are energy-intensive and have high startup and maintenance costing. In these stacks, vegetables and fodder production is done by Hydroponic, under controlled conditions and using balanced nutrient solutions as growth medium. On the other hand, vertical farming is bounded to conceptual designs such as multistoried buildings for farming. This is also very energy and cost intensive. Keeping the socio-economic condition of West Bengal as well as India in mind, there is a need of low cost, low energy consuming stack farming system with locally available materials, so that it can be effectively acceptable by the grass root level farmers. Thus, this was an attempt to design a simple stack to increase the effective farming area. No manipulation of temperature, humidity etc. was done. The set-up was run in the natural field condition (ambient condition) so that the probable problems could be faced.

The experimental set up was designed and performed with multidirectional objectives such as-

- Whether the stack have any direct adverse effect on the major crop yield or not.
- Whether it is possible to get some production on stacks where conditions are little adverse e.g. defuse sunlight, almost no-soil condition etc.
- Yield if possible will be satisfactory or not and whether it will be economically viable.
- Farmers are satisfied or not and whether they want to do it again.
- If this process motivates them towards organic farming.
- Estimate whether stack/ vertical farming can be an alternative livelihood for a farmer's family.

The success of this design of vertical farming may lead to several advantages. Low initial cost and very low maintenance cost is required. Use of water can be easily managed. So, wastage of water can be minimized. Monitoring and maintenance of the stacks, e.g. eliminating grasses and weeds etc. will be easier. So, no inorganic fertilizer and pesticides are used. It will not only result a very high quality food production but also secure the health of the farmers as well as the surrounding environment. Stack can be a good shade for the major crop and protect them from natural adverse conditions e.g. heavy rainfall. For fertile lands, it will not affect the major crop badly. Per area economy generation will be more and on the other hand, if the land is non-fertile in nature e.g. lateritic barren lands of Birbhum-Bankura-Purulia districts of West Bengal, it would be very effective to produce some vegetables and earn some money from it. So, it can be a very good alternative livelihood. By using greenhouses it can also be used in climate smart agriculture. As less or no fertile land is required it can be well accepted by urban people for kitchen garden even on rooftops. So, indirectly the food mile will be reduced. A little modification can be done for production of good quality fodder

in huge scale as it is already being practiced in Australia (2000 kg fodder/ week in 60 Sq. meter area). Using local resources in properly managed way with no such energy demand, pollutions, toxic residues make the process sustainable. Moreover 'reduced food mile' means less combustion of fossil fuel, which in turn supports the system to be greener. In totality, stack farming will produce foods and generate economy in a sustainable manner and can lead us one step ahead towards food security and sustainable development.

2. Materials and Methodology

2.1 Materials:

Several raw meterials were needed to establish the experimental setup. Bamboo logs, nail and Polythene sheat were nesessary for making the srtuctures of the stacks. For nutrisious growth media Paddy Straw, Organic Garbadge, Rice mill ash, vermi compost were properly mixed in a ratio of 4:3:1:1 with very minimum soil and spread over the polythene sheat of each stack. For introducing various species on the stacks, seeds of Dhania, Pea , Tomato, Capsicum, Beans, Spinach and Onion were used.

2.2 Methodology:

Area of the field was 1 Bigha (approximately 190 ft. X 75 ft.). The entire set up was establihed on November, 2012. Winter season was selected as the experiment execution for two resons: availability of different varieties of vegetables and least possibility of natural hazards e.g. storm, heavy rain fall etc.A two storied stack of dimension 10ft X 6ft X 7ft was made (Fig. 1).

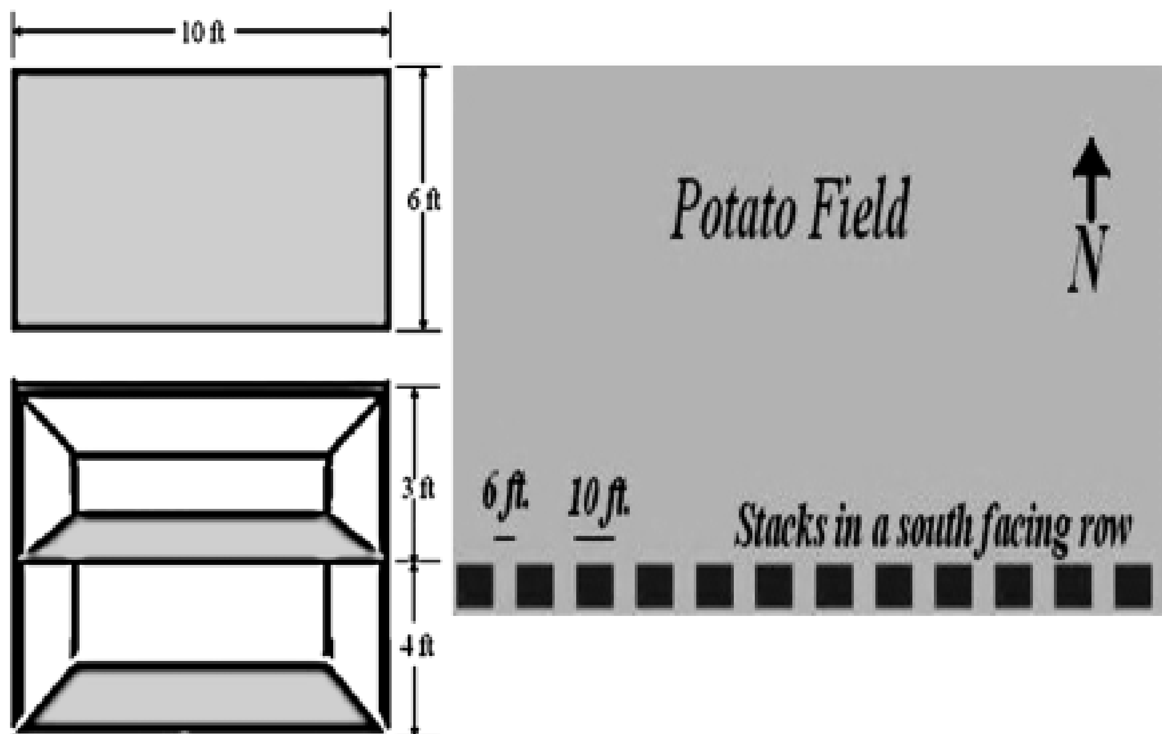


Fig. 1

Fig. 3

First rack was 4ft above ground and second rack was 3ft above the first one. 10 ft side of each stack was kept in east-west direction and 6 ftgap was kept between two consicutive stacks (Fig. 2).

The row of stacks were kept in the southern side of the field for limiting the shadow factor (shadow of those stacks) within the field. Major crop seed (potato) was introduced in the field before stack inoculation. Enough time had been left before stack inoculation so potato plant could be exposed to sunlightat their initial stage of growth.Properly mixed garbage, ash, compost and vermi-compost were prepared. Polythene sheet was spread over each rack. Paddy straw was spread over polythene and over the paddy straw layer the mixture was spread properly. Thickness of the layer was about 2 – 3 inches (Fig. 3). Very little amount of soil was used on the racksso that load (weight) on them could be minimum. All seeds were introduced to both the racks.Short rooted, low water consuming, comparatively high productive,easily available species were selected. After completing the setup, maintenance part was there, that required almost no money and very little time and effort e. g. watering, grass picking etc. Watering was done by sprinkling in regular interval of 2 – 3 days. Eight (8) farmers from three (3) families were involved in the total experiment but that was not their main livelihood. The experiment was terminated on March, 2013 as the major crop was harvested. Soil parameters, like N, P, K, C-N ratio, and porosity were measured through standard methods (APHA, 1995) after completing the experiment. Area allocation for species in middle rack and toprack are shown Fig.4 & Fig.5 respectively.

Fig. 4

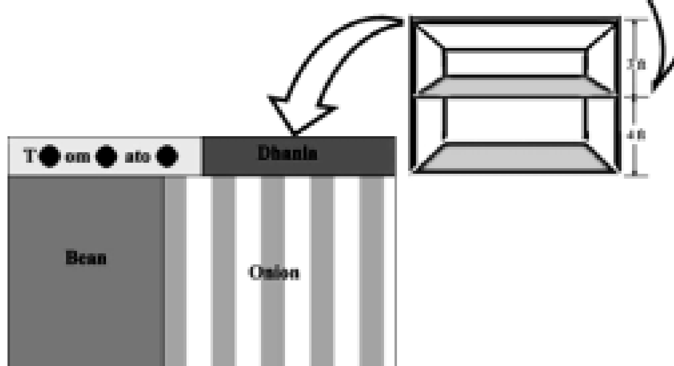


Fig. 5

3. Results and Discussion:

3.1 Soil Parameters:

In case of this experimental set up, use of soil was very less but after completion of the process, the residue of nutrisious growth media was deposited nearby. Samples were collected from that area and were evaluated to determine the N-P-K ratio, C – N ratio and water holding capacity. The N-P-K ratio of a particular soil sample has been measured to determine the fertile condition of it. Nitrogen content of the soil is 31 mg/ L, Phosphate is 23 mg/ L and Potassium is 0.5 – 0.8 me/ 100g. The ratio of total organic carbon (C) and total nitrogen (N) is the traditional guide to determine the soil health by identifying the nature of organic matter present in the soil. Same sample shows C: N = 12:1and good water holding capacity.

As the experimental set up was designed and performed in a total uncertainty and had multidirectional objectives it is better to discuss the results separately, one by one, according to their objectives.

3.2 Whether the stacks have any direct adverse effect on the major crop yield or not:

Area of the potato field was1 *Bigha* or 20 *Kattha* or 14400 Sq. ft. &total area under the stack was 720 Sq. ft. For both setuation, yield per unit area is shown in Fig. 6. Total production of potato (including the area under the stack) was4.636 Ton whereas the same only under the stack was256kg.

Therefore, production outside the stack area is (4636 – 256) kg. =4380kg.Therefore, outside the stack area yield (per hectare) is {4380/(14400-720)*107639} kg/ha= 34.460 Ton/ ha(As, 107639 Sq. ft. = 1 Ha.) and under the stack area yield (per hectare) is (256/ 720*107639) kg / ha = 38.3 Ton/ ha.

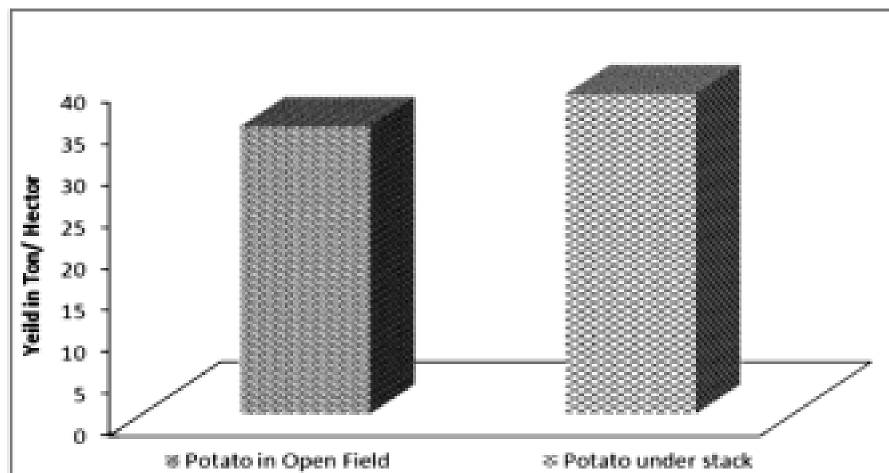


Fig. 6

There was a heavy rain on February, 2013 which had caused a huge damage to the potato in open area. But the area under the stack was safe duo to cover. That's why yield per Sq. ft. is higher for the area under the stack.So, stack farming has no such direct adverse effect on the production of potato (Fig. 7).

Fig. 7

3.3 Whether it is possible to get some production on stacks where conditions are little adverse e.g. defuse sunlight, almost no-soil condition:

The design of the stacks was done in such way that maximum sunlight can be utilized. All the stacks were long in east-west direction and shorter in north- south direction. Pictures (Fig. 8 to Fig. 13) show that, some production was obviously there and that was also with good health.

Dhania and Beans were cultivated in both the stacks. It was observed that for both the species, production and health were much better in the middle stacks. But, for Tomato, the Scenario was totally opposite.

3.4 Whether yield will be satisfactory or not:

Using Stack farming, 1440 Sq. ft. of cultivable area was generated. Production of vegetables on these stacks was quite high. 31.6 kg Beans, 37 kg Dhania, 22.7 kg Onion with 6.7 kg onion leaves and 15.5 kg onion flower sticks, 13.9 kg Pea, 47.8 kg Tomato and 89 kg Spinach were

produced. So, it was possible to produce above 264 kg vegetables with 256 kg Potato, the major crop.

Though, Capsicum was not successfully productive on the stacks, eight plants were in good condition and sifted to ground from stack as the experiment was terminated. Species wise yield per unit area is shown in the following graph (Fig. 14).

The graph (Fig. 14) shows that, among the vegetable species, according to yield per unit area, the rank is Spinach > Tomato >Dhania>Pea > Beans> Onion. As, Nitrogen content was high in the soil, vegetative growth of plants was excellent. That's why production of Spinach and Dhaniawas much above expectation. Maximum standard deviation is found for Tomato as some Tomato plants in middle stacks were not so productive. Standard deviation is also high for Dhania as on the top stack its production was fluctuating in nature.

As these productions were achieved by minimum expenditure and effort and most importantly without hampering the major crop production, it can be concluded that the obtained yield is satisfactory.

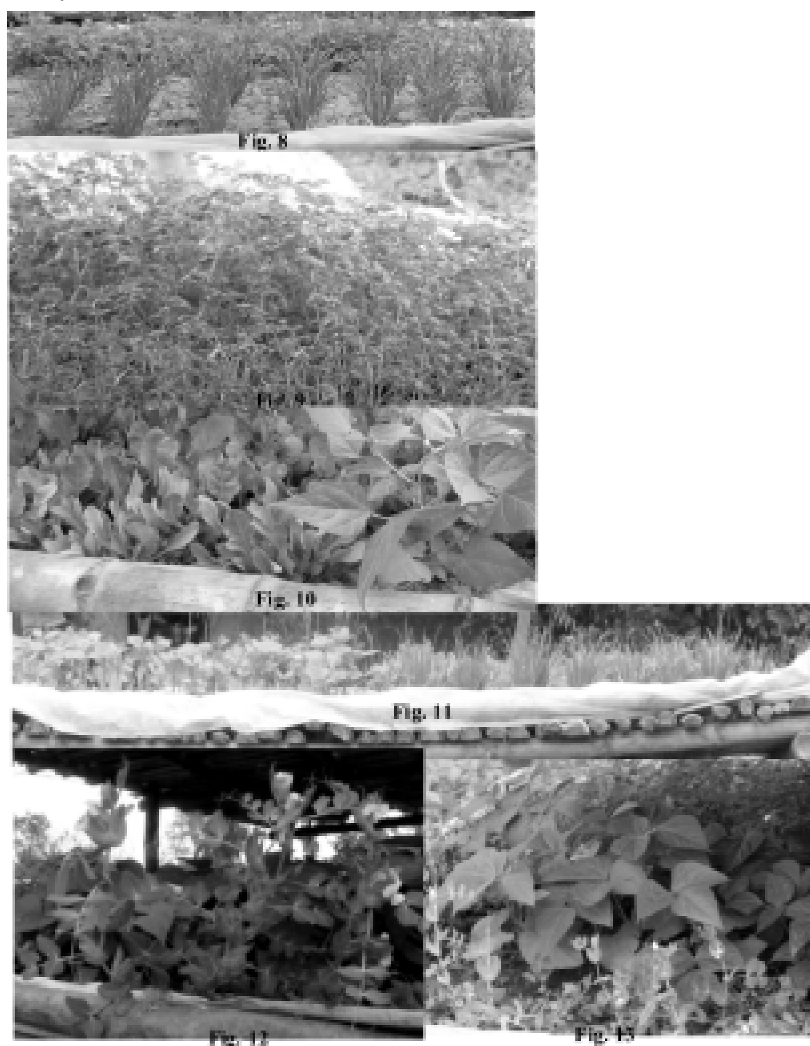


Fig. 8 Fig. 10 Fig. 11 Fig. 12 Fig. 13

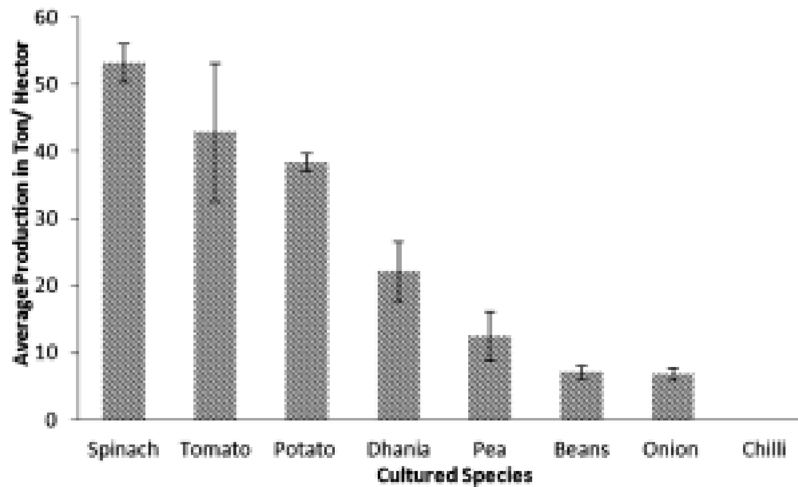


Fig. 14

3.5 Cost Benefit Analysis:

3.5.1 Investment:

For major crop cultivation, total investment was about INR 19, 000. Along with that for cultivation on stack, additional costing was about INR 8, 500 including all kind of expenditures.

3.5.2 Income:

Income of vegetables on these stacks was quite satisfactory. INR 950 from Beans, INR 1480 from Dhania, INR 500 from Onion, INR 8 from onion leaves and INR 310 from onion flower sticks, INR 560 from Pea, INR 2, 390 from Tomato and INR 4, 000 from Spinach. So, it was possible to earn above INR 10, 200 from vegetables with INR 32, 400 from Potato, the major crop.

3.5.3 Profit from Stack Farming:

Profit from major crop, Potato was INR 32, 400 – INR 19, 000 = INR 13, 400. Besides this, whatever profit was obtained was from stack framing. Therefore, gross profit of stack farming is INR10, 270– INR8, 130= INR2, 140.On other words, profit percentage of stack farming becomes {(2140/ 10270)*100} = 20.84%. So, Stack farming can be a good alternative livelihood.

Species wise contribution to this profit is as per the pie graph (Fig. 15). As per the pie graph, Spinach contributes the most (39%) followed by Tomato (23%) and Dhania (14%). Capsicum has no contribution in economy generation; rather, introduction of it was not a good or successful investment.

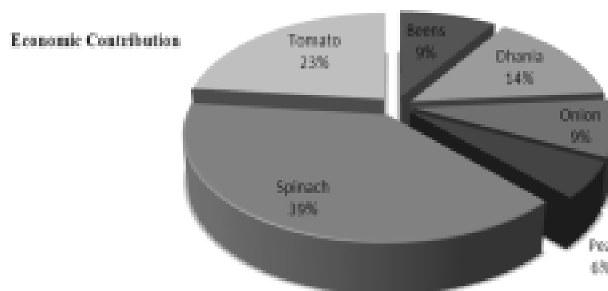


Fig. 15

3.5.4 Farmers' satisfaction:

As the first attempt was successful, eight farmers who were engaged are ready to reset up stacks. They now want to establish bamboo stacks with concrete supporting pillar, so that the structure can remain intact for several years

4. Conclusion:

This experimental set up was established to find answers of many questions. As no funding organization was there, the investment was done in a high risk. It was very difficult to motivate the farmers. Besides all these, finally the production was good, quality was excellent and economic profit was moderate. Some amount of the residue of the stacks was mixed with the soil of kitchen garden making it highly fertile and in the summer it gave good production on local vegetables.

Though, the experiment was more or less successful, it showed that bamboo was not durable supporting pillar for the stacks. PVC pipe or concrete post can be the alternative. But, initial investment is the main barrier; each concrete post will cost minimum INR 1000. So, it will not be affordable or economically viable for a grass root farmer. On the other hand, the price of bamboo has suddenly increased. Some more study on stack farming including design; process etc. is needed to make it a real tool for food security as well as alternative livelihood that will lead the society towards sustainable development.

4.1 Future Scope:

This experiment has an ample scope for future. As not many execution of stack farming are there, it may come out as a non-conventional but potential way to food security, alternate livelihood and foremost sustainable society. Many more execution is needed so that modification can be made to increase its efficiency. Modified Stacks can be used for fodder cultivation and can contribute to integrated farming. Test set up can be run in urban area. With micro water shade management, Stack farming can be successfully executed without any major crop in the barren lateritic region. It may be a solution of cultivation to the people living in the areas where increasing soil salinity is a problem e.g. Sundarban. Considering Indian Socio-economic scenario Ground level work is much more needed in this field.

5. Acknowledgement:

We are grateful to Dept. of Environmental Management (University of Kalyani), Jadavpur University and Institute of Ecotoxicology and Environmental Science, Kolkata for laboratory support, Dr. J. K. Biswas, Dr. Dipayan Deand Prof. K. R. Samaddar for inspiration and guidance and all of them who have given their valuable suggestions, time and effort, moral and financial support throughout the experiment.

Reference:

1. Agro-tecture;http://www.agri-tecture.com/post/17738148042/plantagon-is-an-innovative-vertical-farming#.UbIBIT5RexM, visited 2013-06-07
2. Children´s Rights Portal.;Food Crisis. The unequal distribution of resources: The food crisis explained. http://childrensrightsportal.org/fundamental-rights/food/crisis-explained, visited 2013-05-14
3. Despommier Dickson,; Vertical Farm: The World Is Growing Up, St. Martin's Press, New York, USA, 2010, *320 pp*.
4. Despommier, D.; The Rise of Vertical Farms, Scientific American; Nov2009, Vol. 301 Issue 5, p80-87, 8p

5. Despommier, D.; The Vertical Farm: Feeding the world in the 21st century.2010a, 2nd ed. New York: Thomas Dunne Books/St. Martin's Press.
6. Despommier, D. The vertical farm: controlled environment agriculture carried out in tall buildings would create greater food safety and security for large urban populations, Springer Basel AG 2010
7. Despommier, D.;The Vertical Essay. Department of Environmental Health Sciences Mailman School of Public Health Columbia University. 2012.
8. Despommier, D. Why vertical farms? (Video, pdf) http//www.challengesinverticalfarming.org. March 2012
9. Ellis, J; Agricultural Transparency Reconnecting Urban Centreswith Food Production. Dalhousie University, School of Architecture, Halifax, Nova Scotia, 2012.
10. Germer, J. Sauerborn, *et al*; Sky farming an ecological innovation to enhance global food security. Journal of Consumer Protection and Food Safety 14, 2011
11. Groom, M. Meffee& G. Carroll, C.R.; Principles of Conservation Biology. 2005, 3rd ed., Sinauer Associates, Inc., Sunderland, Massachusetts, USA.
12. http://content.time.com/time/specials/packages/article/0,28804,1934027_1934003_1933961,00.html
13. http://media.clemson.edu/public/restoration/iae/vfarm/student_verticalfarming.pdf
14. http://schools.nyc.gov/NR/rdonlyres/9BCCAE33-2D98-41C8-915D- AF7B702E3315/0/NYCDOE_G9_10_LiteracyScience_VerticalFarming_Final.pdf
15. http://ses.library.usyd.edu.au/bitstream/2123/5539/2/Final1.pdf
16. http://www.arch.columbia.edu/files/gsapp/imceshared/pp2341/Carlson-Lalli-PRESENTATION.pdf
17. http://www.economist.com/node/17647627
18. http://www.fieldrobotics.org/~ssingh/VF/Challenges_in_Vertical_Farming/Schedule_files/TING.pdf
19. http://www.paddymchugh.com/pdfs/Vertical%20Farming%20information.pdf
20. http://www.verticalfarm.com/more?essay1
21. http://www.youtube.com/watch?v=y5i7zn0zt5u
22. Kondo, N. 2012; Horticulture meets Automation, , Challengesinverticalfarming.org
23. KuoGioietta, 2010-2011; Future takes Transcultural Futurist Magazine, 1-3p.
24. Local Food for sustainable Communities, Ecos magazine, Dec-Jan2010, Issue 152, p18-21
25. Malek Al-Chalabi,;*Vertical Farming: Skyscraper Sustainability?* A report by Centre for Environmental Policy, Imperial College London. 2010, 3pp
26. Nelkin, J.; Lessons and challenges in vertical farming from urban, commercial production of greens, Challengesinverticalfarming.org, 2012.
27. Ruthner, O. Apparatus for the artificial cultivation of plants, bacteria, and similar organism. United States Patent Office, USA, 1966;
28. Sharanbir S. Parwinder S., Can cities become self-reliant in food? Center for Urban Environment and Economic Development, The Ohio State University, Wooster, OH 44691, USA, 2011.
29. Simply Hydroponics. http://www.simplyhydro.com, visited 2013-04-11
30. Statemaster Encyclopedia 2013;Aeroponics. www.statemaster.com/encyclopedia/ Aeroponics, visited 2013-04-14
31. Statemaster Encyclopedia; Hydroponics. http://www.statemaster.com/encyclopedia/Hydroponics, visited 2013-04-14
32. Urban agriculture, aquaponics and the post-industrial city, Pratt, E. (Video, pdf) Challengesinverticalfarming.org. March 2012 - 15
33. Vertical Farm. www.verticalfarm.com, visited 2013-05-15.

Badal Bhattacharya,Ph.D., was formerly of the faculty of Engineering and Technology, Jadavpur University, Kolkata, India and retired after thirty six years of service (1969-2005) at Faculty of Engineering & Technology of Jadavpur University. He was a member of Executive Council and Court of Jadavpur University from 2001-2005. He was Guest Faculty (2005-2014) of the Faculty of Engineering & Technology, Jadavpur University and visiting Professor and Research Advisor, Vinayaka Mission University, Salem Tamilnadu, India. He has guided twelve doctoral theses and published over one hundred research papers in National and International Journals. He has also guided number of M. Tech, M. Phil and M.D.S (Orthodontic) students. He has expertise in Nano Engineering, Chemical Process Engineering, Material Engineering and Environmental Engineering. He has also adjudicated many doctoral theses from Foreign and Indian Universities. He is also an Author, Editor and Contributor of an Elsevier B. V. Registered office: Radarweg 29, 1043 NX Amsterdam.

Dr. Bhattacharya is a recipient of Major Research Projects from Department of Science and Technology, Council of Scientific and Industrial Research, All India Council for Technical Education, Board of Research in Nuclear Sciences, Government of India, Department of Science and Technology and Ministry of Mines, Government of West Bengal. He has contributed chapters in books viz, The Ocean: Physical-Chemical Dynamics and Human Impact, Environmental Contaminants Ecosystems and Human Health, Ecology of Wetlands and Associated Systems, Renewable Energy: Trends and Prospects Published by Pennsylvania Academy of Sciences, U.S.A. He was awarded patent by Government of India for his work on recovery of Gallium from bauxite ore during 2007. He has edited number of books on Environmental Sciences. He is the Editor-in –Chief of the Institute of Ecotoxicology & Environmental Sciences. He has visited U.S.A., European Countries, Asia Pacific Countries and South East Asian Countries with various assignments. Dr. Bhattacharya is a Fellow of Indian Chemical Society, Life Member of Indian Association for the Cultivation of Science, Life Member of Association of Corrosion Engineers and Founder President of Institute of Ecotoxicology and Environmental Sciences. He is endowed with one daughter and one son (Sharmistha an E.N. T. Surgeon and Siddhartha an Expert in Sports Medicine). His wife Mrs. Lakshmi Bhattacharya is at present a Retired Teacher in Biology.

Shyamal K. Majumdar, Ph.D., is a Kreider Professor Emeritus of Biology at Lafayette College, Easton, PA, USA. Dr. Majumdar received his M.S. and Ph.D. from the University of Kentucky, Lexington, USA. He has been the editor of Journal of the Pennsylvania Academy of Science for over 39 years and serving on the Editorial board of several national (USA) and international scientific journals. He is the former president of the Pennsylvania Academy of Science (1986-1988) and president of the National Associations of Academies of Science (1991-1992). He is a fellow of the American Association for the Advancement of Science (AAAS) and has authored one book and more than 140 articles and research reports. In addition, he has edited and co-edited 31 books. His research interests include eco- and geno-toxicology, microbiology, cell biology, and genetics.

₹ 650.00 US $ 30 ISBN: 978-81-928924-3-6

View publication stats