EVALUATION OF TRICLOSAN-INDUCED REPRODUCTIVE TOXICITY IN THE FRESHWATER FISH, *Anabas testudineus* (BLOCH, 1792)

Thesis submitted to the

UNIVERSITY OF CALICUT

For the award of the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Sciences

BY

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NOVEMBER, 2021

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CERTIFICATE

This is to certify that Ms. C. V. Priyatha has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph. D ordinance of this University. I recommend her thesis entitled "Evaluation of triclosan-induced reproductive toxicity in the freshwater fish, *Anabas testudineus* (Bloch, 1792)", for submission for the degree of Doctor of Philosophy in this University.

I further certify that this thesis represents the independent work of the candidate under my supervision and no part of the thesis has been presented for the award of any other degree, diploma or associateship in any University.

Dr. K. C. Chitra
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DECLARATION

I hereby declare that the work presented in the thesis entitled "Evaluation of triclosan-induced reproductive toxicity in the freshwater fish, *Anabas testudineus* (Bloch, 1792)" is a genuine record of research work done carried out by me under the guidance and supervision of Dr. K. C. Chitra, Associate Professor, Department of Zoology, University of Calicut. To the best of my knowledge, no part of this thesis has been previously submitted for the award of any degree, diploma or associateship in any other University.

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Dedicated to

The society

ACKNOWLEDGEMENTS

First and foremost my deepest gratitude goes to my beloved supervisor Dr. K.C. Chitra, Associate Professor, Department of Zoology, University of Calicut for her patience, motivation, understanding, encouragement, support, and personal guidance which helped me to perform my work comfortably. She opened a world of enthusiasm and immense knowledge in front of me. It has been an honour for me to be her student. Besides, Dr. K.C. Chitra has introduced me to the Endocrinology and Toxicology Lab family members, Asifa K.P., Vidya P.V., Revathy V., Sumi N., Raibee Mol P., Nikhil John, and Reeha Mashirin K. Their constructive criticisms and discussions, and new insights have been constantly encouraged me when I was stuck during work. I take this opportunity to express my deep and sincere gratitude to my fellow labmates for all their help, support, and considerations.

I thank my parents, Mohanan C.V. and Nalini C. for their selfless actions that made a way ahead to all my accomplishments. Thank you from the deepest part of my heart for all your sacrifices, struggles, and tolerances for helping me to shape my future. I owe all my success to you.

I gratefully acknowledge Dr. Jeena N.S., Scientist, CMFRI, Kochi for her encouragement, and personal attention during my gene expression work in their laboratories and Dr. Sajeela K., Technical assistant, CMFRI, Kochi for sharing her expert qRT-PCR knowledge with me.

I gratefully acknowledge Kerala State Council for Science, Technology, and Environment (KSCSTE), a state-funded agency for encouraging and promoting science and technology-related activities in Kerala, for the research grant that made my Ph.D. works possible.

I am always thankful to my sister, Amrutha Mohanan C.V., for being there for me regardless of my stress levels and for keeping me grounded. For my other half, Dr. Siva Prasad M.S., you have been the most tolerating, loving, and understanding partner throughout our life and I would like to express my deepest gratitude for your endless love, care, and encouragement. I'll never forget the support and kindness given by my in-laws and I am always grateful to them. I would also like to extend a special thank you to my dearest friends Surya P., Sreevidya P., Bindu P.U., and Jalala Najath K. for all the coffee breaks, laughs, and good times.

I thank all the faculty members, non-teaching staff, fellow research scholars, and other members of the Department of Zoology, University of Calicut, for their support and assistance, your kindness will never be forgotten. I also extend my thanks to all those who directly or indirectly contributed to this research. Thank you for everything.

Priyatha C.V.

CONTENTS

ABSTRACT

Triclosan, a lipophilic antimicrobial agent, enters into the aquatic ecosystems through untreated sewages or domestic wastewater effluents. The present study was aimed to evaluate the reproductive toxicity of triclosan at sublethal and environmentally relevant concentrations in the freshwater fish, *Anabas testudineus*. Accordingly, the thesis was structured into seven different chapters. In chapter 1, a general introduction of triclosan, its physico-chemical properties, environmental fate, mode of action, and effects on aquatic organisms, particularly fish was discussed. In chapter 2, four different stages of gonadal development under the laboratory conditions were determined namely preparatory stage from February to March, pre-spawning stage from April to June, spawning stage from July to August, and post-spawning stage from September to January, showing the fecundity rate between 6,500 and 11,000 eggs. Chapter 3 reported the acute toxicity of triclosan using Finney's Probit analysis, which was 1.767 mg L^{-1} concentration, and also elaborated behavioral modifications and histological changes in gill and liver tissues. Chapter 4 discussed the consequences of triclosan at sublethal and environmental concentrations in gonads during the pre-spawning period, which revealed a declined gonadal steroidogenesis, altered aromatase gene expression, modified levels of serum gonadotropins, and sex hormones. Chapter 5 concluded the effects of triclosan during the spawning period, which showed altered biosynthesis of sex steroid hormones by declining the activities of steroidogenic enzymes. Besides, induction of vitellogenin, gonadal lesions, and reduction in sperm motility, viability, and count of male fish along with a decline in the fecundity of female fish indicated that triclosan affected the normal physiology of reproduction. In chapter 6, the induction of oxidative stress in gonads, and genotoxicity in peripheral erythrocytes were demonstrated. Finally, in chapter 7, general conclusions of the present findings were highlighted graphically, which provides better insights on the reproductive toxicity of triclosan in the fish, *A. testudineus*.

സംഗ്രഹം

പലതരം മരുന്നുകളിലും വ്യക്തിപരിചരണ ഉല്പന്നങ്ങളിലും സൂക്ഷ്മജീവികളുടെ വളർച്ചയെ തടയാൻ വേണ്ടി വളരെ സാധാരണയായി ഉപയോഗിക്കുന്ന കൊഴുപ്പിൽ അലിയുന്ന ഒരു രാസവസ്തുവാണ് ട്രൈക്ലോസാൻ. യഥാവിധി പരിപാലിക്കാത്ത ഗാർഹിക/ വ്യാവസായിക മലിനജലം ജലസ്രോതസുകളിലേക്ക് ഒഴുക്കിവിടുന്നത് ട്രൈക്ലോസാന്റെ സാന്നിധ്യം സ്ഥിരമായി ജലസ്രോതസുകളിൽ കാണപ്പെടുവാൻ കാരണമാകുന്നു. മരണകാരണമാകാത്തതും, നമ്മുടെ ജലസ്രോതസുകളിൽ നിന്നും കണ്ടെത്തിയതുമായ അളവുകളിൽ ട്രൈക്ലോസാൻ ശുദ്ധജല മത്സ്യമായ കരിപ്പിടിയുടെ പ്രത്യുൽപാദനവ്യവസ്ഥയെ എങ്ങിനെ ബാധിക്കുന്നു എന്നതാണ് ഏഴ് അധ്യായങ്ങളിലായി ഈ പ്രബന്ധത്തിൽ വിശകലനം ചെയ്യുന്നത്. ആദ്യത്തെ അധ്യായത്തിൽ ട്രൈക്ലോസാനെ കുറിച്ചും പ്രബന്ധത്തിലെ പൊതുവായ പഠനമേഖലകളെ കുറിച്ചും ഹ്രസ്വമായി പ്രതിപാദിക്കുന്നു. അധ്യായം രണ്ടിൽ പരീക്ഷണശാലയിൽ വളർത്തിയ കരിപ്പിടിയുടെ നാല് പ്രത്യുൽപാദന ഘട്ടങ്ങളെ കുറിച്ച് വിശദമാക്കുന്നു. ആദ്യഘട്ടം ഫെബ്രുവരി മുതൽ മാർച്ച് വരെ നീണ്ടുനിൽക്കുന്ന ജനനേന്ദ്രിയത്തിന്റെ വളർച്ചാ ഘട്ടമാണ്. രണ്ടാം ഘട്ടം ഏപ്രിൽ മുതൽ ജൂൺവരെ നീണ്ടു നിൽക്കുന്നതും, പ്രജനനത്തിനു മുന്നോടിയായി കാണപ്പെടുകയും ചെയ്യുന്നു. ജൂലൈ–ആഗസ്റ്റിലായുള്ള മൂന്നാം ഘട്ടത്തിൽ മത്സ്യത്തിന്റെ പ്രജനനം നടക്കുന്നു. ഏകദേശം 6500 മുതൽ 11,000 വരെ മുട്ടകൾ ഈ കാലയളവിൽ കരിപ്പിടി ഉല്പാദിപ്പിക്കുന്നു. എന്നാൽ സെപ്തംബർ മുതൽ ജനുവരി വരെ നീണ്ടുനിൽക്കുന്ന നാലാം ഘട്ടത്തിൽ പ്രത്യുൽപാദന പ്രവർത്തനങ്ങൾ ഒന്നും തന്നെ നടക്കുന്നില്ല. മൂന്നാമധ്യായത്തിൽ ഫിന്നിയുടെ പ്രോബിറ്റ് അപഗ്രഥന രീതി അവലംബിച്ച് കരിപ്പിടിയിൽ ട്രൈക്ലോസാന്റെ അതിതീവ്ര വിഷാംശം ഒരു ലിറ്ററിൽ 1.767 മില്ലിഗ്രാം ആണെന്നു കണ്ടെത്തി. കൂടാതെ ട്രൈക്ലോസാന്റെ വിഷാംശം മത്സ്യത്തിന്റെ സാധാരണ സ്വഭാവത്തിലും ചെകിള, കരൾ എന്നിവയുടെ സ്വാഭാവിക കോശഘടനകളിലും മാറ്റങ്ങൾ വരുത്തുന്നതായി കാണപ്പെട്ടു.

മരണകാരണമാകാത്തതും, നമ്മുടെ ജലസ്രോതസുകളിൽ നിന്നും കണ്ടെത്തിയതുമായ അളവുകളിൽ ട്രൈക്ലോസാന്റെ സാന്നിധ്യം പ്രജനനത്തിനു മുന്നോടിയായുള്ള ഘട്ടത്തിൽ മത്സ്യത്തിന്റെ പ്രത്യുൽപാദനവ്യവസ്ഥയെ എങ്ങിനെ ബാധിക്കുന്നുവെന്ന് അധ്യായം നാലിൽ വിവരിക്കുന്നു. പ്രധാനമായും പ്രത്യുൽപാദന ഹോർമോണുകളുടെ ഉല്പാദന-നിയന്ത്രണ വ്യവസ്ഥയേയും അവയുടെ സാധാരണ അളവിനേയും ട്രൈക്ലോസാൻ കുറക്കുന്നതായി പഠനത്തിൽ കണ്ടെത്തി. എന്നാൽ പ്രജനന ഘട്ടത്തിൽ ട്രൈക്ലോസാന്റെ വിഷാംശം മത്സ്യത്തിന്റെ പ്രത്യുൽപാദന ശേഷിയെ എങ്ങനെ ബാധിക്കുന്നു എന്ന് അധ്യായം അഞ്ചിൽ വിവരിക്കുന്നു. ട്രൈക്ലോസാന്റെ വർദ്ധിച്ച അളവ് പ്രധാനമായും ബീജങ്ങളുടെ എണ്ണം, ചലനം, ജീവൻ, അണ്ഡത്തിന്റെ എണ്ണം എന്നിവയെ ഹാനികരമായി ബാധിക്കുന്നു. കൂടാതെ പ്രത്യുൽപാദന ഹോർമോണുകളുടെയും അവയെ നിയന്ത്രിക്കുന്ന അന്ത:സ്രാവഗ്രന്ഥി ഹോർമോണുകളുടെയും അളവിൽ ഗണ്യമായ കുറവ്, വിറ്റലോജെനിൻ എന്ന മാംസ്യത്തിന്റെ കൂടുതൽ ഉല്പാദനം, അണ്ഡാശയത്തിലെയും, വൃഷ്ണത്തിലെയും കോശങ്ങളുടെ നാശം എന്നിവയും ട്രൈക്ലോസാന്റെ ദോഷഫലമായി കാണപ്പെട്ടു. ട്രൈക്ലോസാൻ പ്രത്യുൽപാദന അവയവങ്ങളിലെ ഓക്സീകരണ -നിരോക്സീകരണ പ്രവർത്തനങ്ങളിൽ ഏറ്റകുറച്ചിലുകൾ സൃഷ്ടിക്കുകയും, മത്സ്യത്തിന്റെ ചുകന്ന രക്താണുക്കളുടെ മർമ്മങ്ങളിൽ ജനിതകനാശത്തിനു കാരണമാകുകയും ചെയ്യുന്നതായി അധ്യായം ആറിലെ പഠനത്തിൽ നിന്നും വ്യക്തമാകുന്നു. ഈ പ്രബന്ധത്തെ ക്രോഡീകരിച്ച് അധ്യായം ഏഴിൽ രേഖപ്പെടുത്തിയിരിക്കുന്ന ചിത്രിത സംഗ്രഹത്തിൽ നിന്നും ട്രൈക്ലോസാൻ കരിപ്പിടിയുടെ പ്രത്യുൽപാദന വ്യവസ്ഥയെയും പ്രത്യുൽപാദന ശേഷിയേയും സാരമായി ബാധിക്കുന്ന രീതികളെ സമഗ്രമായി വിലയിരുത്താൻ സാധിക്കും.

CHAPTER 1

General Introduction

Chapter 1 **General Introduction**

CONTENTS

LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
AhR	Aryl hydrocarbon Receptor
ATP	Adenosine triphosphate
cm	Centimeter
cu.m	Cubic meter
d	Day
DCC	3,5-dichlorocatechol
DCDD	2,8-dichlorodibenzo-p-dioxin
DCP	2,4-dichlorophenol
DNA	Deoxyribonucleic acid
g	Gram
h	Hour
HPLC	High Performance Liquid Chromatography
IUCN	International Union for Conservation of Nature and Natural Resources
K^+	Potassium monocation
Kg	Kilogram
km	Kilometer
L	Litre
log Kow	Octanol-water partition coefficient
mg	Milligram
min	Minutes
ml	Milliliter
mm Hg	Millimeters of mercury
ng	Nanogram
^oC	Degree Celcius
OECD	Organisation for Economic Co-operation and Development
PPCPs	Pharmaceutical and personal care products
USEPA	United States Environmental Protection Agency
USFWS	United States Fish and Wildlife Service
UV	Ultraviolet

1. GENERAL INTRODUCTION

1.1 Emerging environmental contaminants

There is a growing concern that a wide range of chemical compounds reaches the aquatic environment in measurable quantities, and causes detrimental effects on the fish and other aquatic species. Some of the chemical compounds present in the agricultural products, medical drugs, nanomaterials, microplastics, industrial chemicals, food additives, algal toxins, pharmaceutical, and personal care products are the emerging contaminants that affect water quality worldwide. They are released largely from urban, agricultural, industrial and anthropogenic activities, which are usually detected below microgram level in the environment, and potentially suspected to damage the ecosystems and human health (Pereira *et al*., 2015).

The continuous release of these chemicals into the aquatic ecosystem targets different aquatic fauna by the induction of acute toxicity, bioaccumulation, biomagnification, and persistence thereby leading to undesirable long-term effects (Lopes-Serna *et al*., 2012). The United States Environmental Protection Agency defined the 'emerging contaminant' as '*any synthetic or naturally occurring chemicals or microorganisms that are not commonly monitored in the environment but have the potential, or real threat to the human health or the environment, for which the published health standards are lacking*' (USEPA, 2008).

The list of emerging contaminants is rapidly growing in recent years with reported consequences to organisms such as endocrine disruption, immunotoxicity, carcinogenicity, developmental and reproductive defects, and so on. The toxicity of the contaminants varies among the exposed organisms based on several factors such as physico-chemical properties of the compounds, their distribution in different compartments like air, water, soil, sediments, etc., and biological targets including fish, reptiles, birds, mammals, and humans. Among the different emerging environmental contaminants, the impact of pharmaceutical and personal care products on the aquatic environment is one of the crucial concerns among ecotoxicologists.

1.2 Pharmaceutical and personal care products (PPCPs)

Pharmaceuticals are classes of chemicals with unique physio-chemical properties and biological functions useful for diagnosis, healing, curing or reducing, and preventing any kind of diseases in humans and animals. Based on the therapeutic purpose, these chemicals are classified as antibiotics, palliative, hormones, analeptic, anti-inflammatory, anti-epileptic, anti-coagulant, and cytostatic drugs as well as blood lipid regulators, β-blockers, and contrast medium (Liu and Wong, 2013). Many of the pharmaceutical drugs are not effectively removed during the wastewater treatment processes hence they are largely released into the water bodies, which are prioritized as the emerging contaminants for over 20 years (Miller *et al*., 2018). Meanwhile, personal care products are widely used in several consumer products in the form of antimicrobial agents, synthetic

musks, insect repellants, preservatives, sunscreen UV filters in soaps, shampoos, lotions, toothpaste, fragrances, sunscreens, etc. (Corcoran *et al*., 2010).

PPCPs are the diverse group of chemical compounds commonly used in fragrances are phthalates, parabens, silicones, styrenes, etc., while some include synthetic hormones like ethinylestradiol, and diethylstilbestrol (Dodgen *et al*., 2017). The other categories of chemicals such as nonylphenol, bisphenol A, etc. are used as surfactants or detergents. Some compounds possessing antimicrobial, anti-analgesic properties like triclosan, oseltamivir, diclofenac, ibuprofen, carbamazepine, diazepam, camphor, and so on, along with certain flame retardants, pesticides, biocides, and other inorganic solvents are grouped under the PPCPs as the emerging contaminants (Kinney *et al*., 2006). Most of these compounds reach various compartments of the aquatic environment at low concentrations that ultimately cause detrimental effects to the aquatic biota (Boyd *et al*., 2004).

The mode of entry of PPCPs into the aquatic bodies occurs through multiple pathways including untreated wastewater treatment plants, accidental discharge from the manufacturing sites, disposal from veterinary, pets, livestock, and aquaculture products, and as direct runoff from the agricultural fields (Boxall *et al*., 2003; Martinez-Carballo *et al*., 2007; Overturf *et al*., 2015). However, the mode of action of PPCPs depends on the concentration, degree of metabolism, and rate of detoxification in the exposed organism (Richardson and Bowron, 1985). A large number of PPCPs have been identified as

endocrine disruptors that are known to possess the properties to alter or mimic the endocrine system pathways (Archer *et al*., 2017).

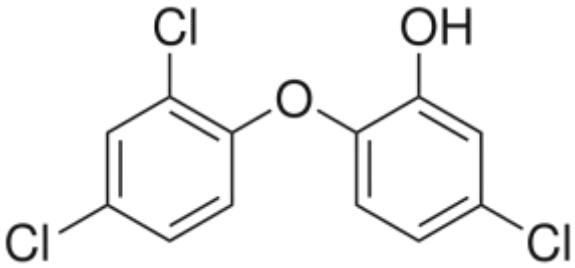
1.3 Triclosan

Triclosan is a broad-spectrum, chlorinated aromatic compound, and used as a pesticide since 1969. Triclosan is known in different brand names such as Irgasan DP300, Cloxifenolum, Triclosanum, Aquasept, Gamophen, Sapoderm, Ster-Zac, etc. (Dann and Hontela, 2011). In the 1970s, owing to its antimicrobial properties triclosan has been used in surgical scrub at hospitals, and as a common ingredient in soaps, antiseptic lotions, shampoos, detergents, mouthwashes, and other cleaning products (Bedoux *et al*., 2012). The European Union Scientific Committee on Consumer Products has approved up to 0.3% concentration of triclosan in cosmetic products, however, its aggregate exposure from all cosmetics was considered unsafe from a toxicological point of view (Dann and Hontela, 2011).

The lipophilicity of triclosan and the presence of both phenol and ether functional groups suggest its bioaccumulation in fatty tissues (Olaniyan *et al*., 2016). Besides, triclosan also has structural similarities with other endocrine disrupting compounds such as polychlorinated biphenyls, polybrominated diphenyl ethers, bisphenol A, diethylstilbestrol, dioxins, and thyroxine (Crofton *et al*., 2007; Allmyr *et al*., 2006). The estrogenic property of triclosan has been associated with endocrine disrupting effects (Raut and Angus, 2010). Other possible health effects include increasing rates of allergies and skin irritability, antibiotic resistance, and the formation of carcinogenic

by-products (Schweizer, 2001; Adolfsson-Erici *et al*., 2002; Russell, 2003).

1.3.1 Structure of triclosan



1.3.2 Chemical and physical properties of triclosan

IUPAC name	:	5-chloro-2-(2, 4-dichlorophenoxy) phenol
CAS number	:	3380-34-5
Trade names	:	Irgasan, Ster-Zac, Tinosan AM110, Invasan DP 300R, 300TEX, Irgaguard RB1000, VIV20, Irgacare MP, Lexol300, Cloxifenolum, Aquasept, Gamophen, Vinyzene DP7000, Microbanish R, Vikol THP
Molecular formula and Molecular weight	:	$C1_2H_7Cl_3O_2$; 289.54
Color	:	White to off-white crystalline, slightly aromatic powder
Specific gravity	:	1.55×10^3 Kg. cu.m^{-1} at 22 ºC

Melting point	:	55–57 ºC
Boiling point	:	280-290 °C
Vapour pressure	:	4×10^{-6} mm Hg at 20 ºC
Thermal decomposition	:	280–290 ºC; pKa = 7.9
Octanol-water partition coefficient	:	(log Kow) 4.76
Bioconcentration factor (BCF) - aquatic organisms	:	2.7–90
Half-life in freshwater	:	2 to 2000 d
Biodegradation (half-life in aerobic soil)	:	18 d
Biodegradation (in anaerobic condition)	:	70 d
Vapour pressure	:	4×10^{-6} mm Hg at 20 ºC
Solubility in water	:	10 mg L^{-1} (20 ºC) - 12 mg L^{-1} (25 °C)
Solubility in organic solvents	:	Methanol, ethanol, acetone, propylene glycol, Tween 20, benzene, dimethyl sulfoxide

Source: https://pubchem.ncbi.nlm.nih.gov/compound/Triclosan; Dhillon *et al*., 2015.

1.3.3 Occurrence of triclosan - Indian scenario

Triclosan is one of the commonly detected PPCPs in the aquatic environment as it has been detected in different compartments like sediments and surface of freshwater and marine ecosystems, and

estuaries (Hontela and Habibi, 2013; Dhillon *et al*., 2015; Xu *et al*., 2020). Triclosan concentrations in aquatic ecosystems depend on the influence of multiple factors such as the nature of effluent or byproducts discharged, rate of degradation, physico-chemical properties, sediment density, organic matter content, the velocity of water current, depth, season, temperature, and pH of the aquatic medium (Reiss *et al*., 2002). Triclosan exists in the surface water of rivers, streams, and lakes at concentrations ranging from 0.0014 to 40 µg L^{-1} while in the wastewater treatment effluents it ranged between 0.023-5.37 µg L^{-1} concentrations (Dhillon *et al*., 2015).

The occurrence of triclosan at nearly 4-10% has been reported from the out-flowing water of wastewater treatment plants (WWTP), among which about 6% exist in the form of biosolids (Singer *et al*., 2002; Bester, 2003). The presence of triclosan in sediment, water, and fishes of Indian rivers, reservoirs, and estuaries varied from nanogram to milligram level (Ramaswamy *et al*., 2011; Jeyakumar *et al*., 2012; Shanmugam *et al*., 2014; Nag *et al*., 2018). Water samples collected from 29 sites along the river Kaveri, Vellar, and Tamiraparani, Tamil Nadu, India showed a higher concentration of triclosan in Tamiraparani river at 5,160 ng L^{-1} concentration followed by Kaveri and Vellar rivers at 40.7 and 8.95 ng L^{-1} concentrations, respectively. Meanwhile, the sediments showed the distribution of triclosan from 11-85.3 ng g^{-1} in river Kaveri, 14-46.87 ng g^{-1} in river Tamiraparani, and the concentration varied between 22.5 and 32.1 ng g^{-1} in river Vellar (Ramaswamy *et al*., 2011).

The surface sediments collected from 15 sites of the Valliyar estuary, Kanyakumari district, Tamil Nadu, India showed the occurrence of triclosan ranged from 132 to 3,073 ng g^{-1} concentration (Jeyakumar *et al*., 2012). Triclosan extracted from the tissues of fish collected from the river Kaveri of Tamil Nadu and Karnataka provinces detected the highest concentration of 50 ng g^{-1} wet weight from Mettur dam due to the high anthropogenic activities, while the low concentration was observed in Kallanai reservoir (Shanmugam *et al*., 2014). Water, sediment, and fish samples collected using isocratic reversed-phase HPLC, from the stretch of about 450 km of River Gomti, a major tributary of River Ganga, India found triclosan between the range of 1.1-9.65 µg L^{-1} in water samples, at 5.11-50.36 µg Kg^{-1} in sediments, and between 13 and 1,040 µg Kg^{-1} wet weight in the tissues of fishes (Nag *et al*., 2018).

The concentration of triclosan detected in the Versova sea-creek interface of Mumbai, India ranged at 1.03-14.5 µg L^{-1} in water samples, and 0.75 µg L^{-1} to 0.38 mg L^{-1} in sediments, respectively (Thilakan *et al*., 2019). A study conducted in five different sites of river Torsa, West Bengal, India has detected its occurrence at 0.055-0.184 µg L^{-1} concentrations in the water samples, while the concentration of residues and metabolites of triclosan ranged between 91.1 and 589 µg Kg^{-1} in about 26 different fish samples (Sarkar *et al*., 2020). The literature reviewed stated that owing to the lack of proper wastewater treatment, a large amount of triclosan is being released continuously in the water bodies of Indian cities. Some criteria and regulations have been put forth by the Central Pollution Control Board

of India to effectively rule out the discharge of untreated industrial effluents and domestic wastewater into the Indian rivers, but remain in its infancy. Concurrently, studies on the persistence and toxic effects of PPCPs and endocrine disruptors like triclosan have gained less attention in the Indian scenario.

1.3.4 Toxicokinetics of triclosan

The physico-chemical properties of triclosan demonstrate its ability to persist in the environment and bioaccumulate in the exposed organisms. Triclosan is readily absorbed through the mucosa, skin, and by oral ingestion to reach the gastrointestinal tract, which is later detected in the systemic circulation, and the high concentrations were noticed in the liver tissue, followed by adipose tissue and brain (Geens *et al*., 2012). Triclosan interacts with the erythrocyte cell membrane causing K^+ leakage and membrane damage associated with a decrease in total erythrocyte count, hemoglobin content, and packed cell volume while increases the total leukocyte count (Sahu *et al*., 2018). The biotransformation of triclosan forms several metabolites including 4-chlorocatechol, 2,4-dichlorophenol (DCP), 3,5-dichlorocatechol (DCC), 2,8-dichlorodibenzo-p-dioxin (DCDD), and monohydroxyl-triclosan by ether cleavage, cyclization, and hydroxylation in the phase I reaction (Zhu *et al*., 2018).

Cytochrome P450 enzyme system catalyzes the phase I metabolic reaction, and the metabolites formed were found more toxic than the parent compound with typical AhR mediated toxic effects (Ku *et al*., 2014). The second step of biotransformation involves phase II

reaction, in which triclosan undergoes detoxification by the addition of highly charged polar moiety to hydroxyl groups through glucuronidation and sulfonation reactions (Wu *et al*., 2010; James *et al*., 2012). The major conjugated metabolites of triclosan formed in the phase II reactions such as triclosan sulfate, triclosan glucuronide, 2,4-dichlorophenol, and hydroxytriclosan are readily eliminated through feces and urine (Rodricks *et al*., 2010).

1.3.5 Fate of triclosan in humans

Humans are exposed to triclosan directly from the use of consumer products, ingestion of plants grown in soil containing triclosan sludge or effluents, or by the consumption of the fish exposed to the toxicant. Likewise, rapid dermal absorption of triclosan of approximately 3-7% from the cosmetic products, and about 7.33 % by the use of mouthwash on reaching the gastrointestinal tract attains high concentration (Bagley and Lin, 2000). Thus humans subjected to triclosan through direct or indirect pathway contribute its presence at the variable amount in the body fluids. The presence of triclosan has been identified in human samples such as urine (Calafat *et al*., 2008; Yin *et al*., 2016), blood plasma (Allmyr *et al*., 2008), and breast milk (Allmyr *et al*., 2006) indicating the direct absorption from the consumer products.

The total median concentration of triclosan in the maternal blood plasma and amniotic fluid in Indian women were found to be 7.17 ng ml^{-1} and 7.04 ng ml^{-1}, respectively (Shekhar *et al*., 2017). However, the level of triclosan concentration in the pregnant women

and their fetuses of Beijing and Qingyuan city, China were detected with comparatively low concentration in the maternal serum at 1.9 ng ml^{-1} and cord serum at 2 ng ml^{-1} than the Indian women (Bai *et al*., 2020). Meanwhile, the presence of triclosan in fetuses and amniotic fluid reveals that the substance is likely to pass the placental barrier between the mother and child. Triclosan is lipophilic and relatively stable, therefore, the presence is noticed in the human milk (Ito and Lee, 2003), and also cause considerable risk to human babies on the consumption of milk from triclosan-exposed mother (Arcus-Garth *et al*., 2005).

A study conducted in California and Texas found that human breast milk contains triclosan at concentrations ranging from 2-100 µg Kg^{-1} lipid, which concerns that its presence even at a low amount in human breast milk could cause risk to infants (Dayan, 2007). A randomly selected population from Sweden and United States found that the excretion of triclosan in urine occurs at 743 µg and 3,790 µg per litre per day indicating urinary excretion as the major route of triclosan elimination, while the fecal elimination is only the secondary route (Fang *et al*., 2010). Humans subjected to triclosan consumption increased the urinary excretion within the first 24 h, and the remaining 24-83% excreted within 4 d, which later approached the baseline levels within 8 d of the initial exposure, predominantly in the form of conjugates (Lin, 2000).

Triclosan exhibits endocrine disruption activity in humans thereby leads to adverse reproductive effects such as cryptorchidism, hypospadias, precocious puberty, dermal allergies, apoptosis, immune

dysfunction, anti-thyroid activity, and carcinogenicity (Olaniyan *et al*., 2016). Several *in vitro* studies on human cell lines also demonstrated the adverse health effects of triclosan by mitochondrial uncoupling resulting in the reduction of mitochondrial ATP production in primary human keratinocytes (Ajao *et al*., 2015; Weatherly *et al*., 2016). Besides, proliferation of the breast cancer cells, apoptosis and cell membrane damage in lung cancer cells, loss of mitochondrial transmembrane potential, metabolic acidosis, and uncoupling of oxidative phosphorylation also occurs in various cell lines (Henry and Fair, 2013; Kwon *et al*., 2013) indicating the exposure through various consumer products is sufficient to evoke adverse effects in humans.

1.3.6 Triclosan on aquatic organisms

Triclosan, an antimicrobial agent, is frequently detected in the aquatic compartments from untreated wastewater treatment plants. Photodegradation of triclosan to 2,8-dichlorodibenzo-p-dioxin (DCDD) and other dioxin derivatives occurs in the aquatic environment. The metabolites or biotransformation products of triclosan are often more toxic than the parent compound (Ding *et al*., 2018). The occurrence of triclosan in the wastewater, sewage sludge, surface waters, and sediments leads to bioaccumulation in freshwater and marine organisms. Thus triclosan is highly toxic to aquatic fauna, where the microorganisms, algae, invertebrates, and fish are equally targeted (Orvos *et al*., 2002). Microalgae exhibit high sensitivity to triclosan as it is known to affect growth, glucose metabolism, failure of survival, and reproduction (Tatarazako *et al*., 2004).

Daphnia magna exposed to triclosan at 330 µg L^{-1} for 48 h increased sensitivities of superoxide dismutase, malondialdehyde, and aminopyrine N-demethylase (Peng *et al*., 2013). In zebra mussels, triclosan affects the activity of oxidases and induced DNA damage possibly leading to cytotoxic and genotoxic effects (Binelli *et al*., 2009). Similarly, triclosan exerts cytogenotoxic effects in the freshwater mussel *Dreissena polymorpha* by the induction of oxidative stress and altered protein expression profiles in gills stating the stress response in the aquatic non-target organism (Riva *et al*., 2012). Exposure of triclosan alters the developmental health of fish, particularly causing edema in the pericardium and yolk sac, impairs hatching success and growth rate in larval zebrafish (Oliveira *et al*., 2009), and also alters swimming behavior, skeletal malformations, and other behavioral patterns in Japanese medaka and fathead minnow (Nassef *et al*., 2010; Schultz *et al*., 2012). Developmental sublethal toxicity of triclosan causes blue sac syndrome by disrupting calcium homeostasis in cardiac muscle followed by the reduction in heart rate, alteration in the relaxation of heart tissue, cardiac edema, hemorrhage, and craniofacial malformations (Saley *et al*., 2016). Among the aquatic organisms, fish are more exposed to contaminants as they occupy at the top of the food chain where the toxic compounds end up more and more, and also they are exposed directly from the surrounding environment.

1.4 Fish as an aquatic bioindicator species

Chapter 1: General Introduction

Fishes are the most diverse species that occupy both marine and freshwater habitats. The information extracted from FishBase as of September 2020 recorded about 34,300 fish species globally, which is more than the combined total of all other vertebrate species including amphibians, reptiles, birds, and mammals. Owing to the richness and diversity of fish species, any change in the water quality directly affects the health status of the population. Fish are highly sensitive to a wide range of environmental stressors so that they are extensively used as a precise bioindicator in ecological, physiological, and toxicological research. Bioindicator organisms are known in different names such as sentinels, keystones, flagships, ecological indicators, vulnerable, umbrellas, and so on (Noss, 1990).

Fish are used in ecotoxicological studies to monitor the quality of the ecosystem, habitat alterations, and the presence and adverse effects of various pollutants. Some of the most commonly used and selective indicator species in toxicology studies include zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), trout (*Oncorhynchus mykiss*), and fathead minnow (*Pimephales promelas*). Besides, other fish species like Atlantic killifish or mummichog (*Fundulus heteroclitus*), goldfish (*Carassius auratus*), roach (*Rutilus rutilus*), pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*), swordtail (*Xiphophorus hellerii*), three-spined stickleback (*Gasterosteus aculeatus*), mozambique tilapia (*Oreochromis mossambicus*), orange chromid (*Pseudetroplus maculatus*), mosquitofish (*Gambusia affinis*), climbing perch (*Anabas testudineus*), etc., are widely used as laboratory models (Ribas and Piferrer, 2014). Selection of bioindicator

species as test model requires certain characteristic properties like species abundance, good dispersal mechanism, high reproductive capacity, easy handling, adaptation in cage culture, and tolerance to pollutants (Leung *et al*., 2000).

Zebrafish are used as an animal model since the 1960s in several biological research including drug toxicity, biomedical research, developmental and reproductive biology, and genetics (He *et al*., 2014). The transparent body of the fish during the larval developmental stage best suits as the model for embryonic studies, and approximately 80% of the genes involved in human diseases are found equivalent in zebrafish (Langheinrich *et al*., 2002). Medaka has many attributes that are similar to zebrafish and is emerging as an important model organism mainly for carcinogenic, toxicology, and genetic studies (Wittbrodt *et al*., 2002). However, the rainbow trout replaced the small fish models like zebrafish and medaka in various fields of research such as toxicology, comparative immunology, carcinogenesis, physiology, and evolutionary genetics because of its large size, inexpensive culture, long reproductive cycles, and well established genomic information (Bailey *et al*., 1996).

Recently, most of the toxicogenomic analysis was performed using fathead minnow as the laboratory model since it possesses many qualities like temperature tolerance, high adaptability in controlled experimental conditions, well-defined reproductive and developmental cycles, easy culture in the laboratory, and high spawning rate (Saari *et al*., 2016). The detailed knowledge of the morphological and physiological aspects of different fish species helps to select suitable

fish as a model for specific issues of concern (Chovanec *et al*., 2003). There are some criteria to be fulfilled for the selection of model organisms in research, which include small size, easy maintenance and culture in the laboratory, responsiveness, more longevity, nutritional value, and more informational resources (Dietrich *et al*., 2020). The present study selected the native species *Anabas testudineus* as the model organism and is widely used as bioindicator species in many toxicology studies.

1.5 *Anabas testudineus* (Bloch, 1792) - the laboratory model

Anabas testudineus (Bloch, 1792)

1.5.1 Species profile of Climbing perch - *Anabas testudineus*

Kingdom	:	Animalia
Subkingdom	:	Bilateria
Infrakingdom	:	Deuterostomia
Phylum	:	Chordata
Subphylum	:	Vertebrata
Infraphylum	:	Gnathostomata

Superclass	:	Actinopterygii
Class	:	Teleostei
Superorder	:	Acanthopterygii
Order	:	Perciformes
Suborder	:	Anabantoidei
Family	:	Anabantidae
Genus	:	Anabas (Cloquet, 1816)
Species	:	*Anabas testudineus* (Bloch, 1792)

Common name - Climbing perch, Climbing bass, Climbing gourami

Anabas in Malayalam language - Chempally, Kalladamutty, Karuveppu, Karippidi, Kallada, Antikalli

Source: ITIS Standard Report Page: *Anabas testudineus*

1.5.2 Characteristics of *Anabas testudineus*

Anabas testudineus is an economically important, obligatory air-breathing teleost fish that inhabits South-East Asian countries. The International Union for Conservation of Nature and Natural Resources (IUCN) has categorized *Anabas testudineus* as a vulnerable species (Singh *et al*., 2012). It comprises four genera with 33 species, mostly lives in freshwater or brackish water like ponds, swamps, canals, lakes, and estuaries, and is an omnivorous feeder (Norris and Douglas, 1992). The natural food for the fry of the fish includes phytoplankton and zooplankton, while the adults feed on algae, worms, crustaceans, mollusks, soft higher plants, insects, and organic debris (Bhattacharjee and Chandra, 2016). The presence of an accessory air-breathing organ

helps the fish to tolerate extremely unfavorable water conditions. Moisture in the air-breathing organs makes them survive out of water for several days or weeks, and they keep crawling or moving over the land between water bodies using their highly mobile suboperculum and strong spiny fins (Binoy, 2015).

The body of the fish is elongated and laterally compressed having fairly a large mouth with villiform teeth on jaws. Body color is dark to pale greenish, it fades to pale yellow on belly region with dark grey colored dorsal and caudal fins, pale yellow pectoral and anal fins, and pale orange pelvic fins. However, the color pattern differs considerably in different localities according to the change in environmental conditions. Pectorals and caudal fins are round while the dorsal, pelvic and anal fin rays are modified to spines (Pethiyagoda, 1991). The dorsal fin possess16-18 spines and 8-10 soft rays, the anal fin has 8-11 spines and 9-11 soft rays, the pectoral fin with 13-14 soft rays, and pelvic or ventral fin is single spined with five soft rays. Dark spots are found at the operculum near to pectoral fin, and also at the base of the caudal region, which fades with age (Pethiyagoda, 1991).

The nutritional value of the muscle tissue of *A. testudineus* shows a seasonal variation where the high value of protein and lipid at 17.07% and 14.06%, respectively, were observed during the March and April months (Monalisa *et al*., 2013). *A. testudineus* is bisexual, but sexual dimorphism is observed only during the breeding season based on the differences in body shape and fin color (Mookerjee and Mazumdar, 1946; USFWS, 2014). Besides, it is a seasonal and annual breeder that breeds only once a year, between April to October in India,

Bangladesh, Thailand, Philippines, etc. (Taki, 1978; Vidthayanon, 2002). The reproductive cycle is short of 3-6 months, in which the fish attains maturity after hatching (Saha *et al*., 2009).

The characteristics of *A. testudineus* that permits to choose as an animal model in research includes (a) native species, (b) abundance in the natural environment, (c) easy maintenance in the laboratory condition, (d) resistance to adverse environmental conditions, (e) shares wide range of habitats, (f) short breeding season, (g) external fertilization, (g) non-adhesive eggs, (h) optically transparent fertilized eggs for easy embryonic studies, and (i) rapid embryonic development. Hence *Anabas testudineus* was widely used as an animal model in various researches including behavior, ecotoxicology, developmental biology, genetics, and reproductive physiology (Tam *et al*., 2015; Rishin *et al*., 2019; Sumi and Chitra, 2020).

1.6 Research focus

The present study aimed to focus mainly on the reproductive toxicity induced by one of the antimicrobial agents, triclosan, in the freshwater fish *Anabas testudineus*. Accordingly, the research plan was executed in different phases namely acute toxicity testing, selection of test concentrations and reproductive stages, followed by the identification of reproductive toxicity in pre-spawning and spawning stages of the fish at sublethal and environmentally relevant concentrations. Finally, an assessment of reproductive impairment in the spawning stage of the fish using some selective reproductive

parameters, histology, genetic damage and oxidative stress were used as the reliable endpoints.

The first phase of this study was mainly targeted to identify the pre-spawning, spawning, and post-spawning stages of the fish in the controlled laboratory condition to perform the toxicity studies. In the second phase, the median lethal concentration of triclosan in the fish, *Anabas testudineus* was determined for the selection of sublethal concentration. Besides, based on the available literature survey, the selection of environmentally relevant concentrations in the Indian scenario was performed.

In the third phase, reproductive toxicity in the pre-spawning phase was evaluated after 90 d of triclosan exposure whereas, in the fourth phase, the study was conducted in the spawning phase to evaluate the reproductive potential of the fish after triclosan exposure. Finally, in the fifth phase, the study aimed to analyze the effect of triclosan in the gonads using oxidative stress and genetic damage as biomarkers in the fish, *Anabas testudineus*. The endpoints monitored throughout the study were followed according to the guidelines prescribed by the Organization for Economic Co-operation and Development (OECD, 2018).

1.7 Objectives of the study

In the past few decades, several endocrine-disrupting chemicals are largely released into the aquatic environment and are referred to as the emerging contaminants. Anthropogenic activities provoke adverse effects on the aquatic environment that affect the aquatic organisms.

Meanwhile, world per capita fish consumption increased tremendously in parallel to the threat of aquatic ecosystems, which concerns the ecotoxicologists in recent years. The research literature pile up the fact that environmental contaminants in the aquatic ecosystem have the potential to disrupt the normal reproduction in fish, which may seriously affect the spawning and fecundity of fish thereby decline its population. The present study, therefore, aimed to investigate the reproductive toxicity of triclosan, an emerging endocrine disruptor in the freshwater fish, *Anabas testudineus*. This study mainly focused on the following objectives to relate the adverse effects on the well-being of humans since the toxicant enters through the food chain.

- ✓ To assess the acute toxicity of triclosan on *Anabas testudineus* by probit analysis.
- ✓ To evaluate the effect of triclosan on steroidogenesis in gonads of *A. testudineus*.
- ✓ To investigate the effects of triclosan on male and female reproduction in the fish.
- ✓ To evaluate hormonal influence of triclosan in fish.
- ✓ To analyse histopathological modification in gonads induced by triclosan.
- ✓ To determine the genotoxic effects of triclosan in fish.

1.8 Thesis outline

The objectives of the study that are discussed in the previous section are categorized into five main chapters, followed by the general conclusions as structured below:

Chapter 2 – Molecular identification of *Anabas testudineus* and assessment of the gonadal stages during development under controlled condition

Chapter 3 – Acute toxicity of triclosan in the fish, *Anabas testudineus*

Chapter 4 – Chronic effect of triclosan on the gonadal development in the pre-spawning phase of *Anabas testudineus*

Chapter 5 – Influence of triclosan on the reproductive physiology of *Anabas testudineus* during the spawning phase

Chapter 6 – Assessment of triclosan-induced oxidative stress and genotoxicity as biomarkers in the fish *Anabas testudineus*

Chapter 7 – General conclusions

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CHAPTER 2

Molecular identification of *Anabas testudineus* and assessment of the gonadal development under controlled condition

Chapter 2 Molecular identification of *Anabas testudineus* and assessment of gonadal development under controlled conditions

CONTENTS

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
°N	Degree North
°S	Degree South
µg L^{-1}	Microgram per Litre
µm	Micrometers
µM	Micromol
ANOVA	Analysis of Variance
APHA	The American Public Health Association
cm	Centimeter
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DMSO	Dimethyl sulfoxide
E2	Estradiol
ELISA	Enzyme-linked immunosorbent assay
F1	First generation
F2	Second generation
Fig.	Figure
FSH	Follicle-stimulating hormone
g	Gram
GSI	Gonado-somatic index
h	Hour
HPG	Hypothalamus-pituitary-gonadal
HSI	Hepato-somatic index
LH	Luteinizing hormone
mg	Milligram
mg L^{-1}	Milligram per litre
min	Minute
mL	Millilitre
mm	Millimetre
ng	Nanogram
OECD	The Organization for Economic Co-operation and Development
P	Probability value

pH	Potential of hydrogen
PRE	Preparatory stage
PRP	Pre-spawning stage
PSP	Post-spawning stage
r	Correlation coefficient
R^2	Square of the correlation
SD	Standard deviation
SP	Spawning stage
SPSS	Statistical Package for the Social Sciences
USA	United State of America

LIST OF FIGURES AND TABLES

1. INTRODUCTION

1.1 Reproduction in fish

Fishes are the most abundant and dominating aquatic vertebrates that encompass nearly 36,000 recognized species globally. Ichthyologists have classified fishes into three major categories based on the nature of skeleton, presence of scales, and the basis of accommodation. As one of the most diverse aquatic vertebrates, they show great variations in species richness, abundance, and phylogeny. Based on salinity tolerance, they are classified into freshwater fish, brackish water fish, and marine fish. They remain successful in all aquatic compartments irrespective of physicochemical features of the water influenced by several factors including light, temperature, presence of phytoplankton causing algal bloom, and contaminants that affect the water quality (Wang and Guo, 2019). Besides, the diversity of fish species are also maintained by their complex life cycle patterns, reproductive and adaptive strategies that allow them to resist and flourish in extreme environmental conditions.

Fish reproduction is considered as one of the crucial and complex physiological processes by which the existence of the species in the natural habitat is maintained. Teleost fish are gonochoristic that exhibit several strategies to maintain their population in response to the sensitive changes in the environment. Sex determination occurs either genetically, or influenced by environmental conditions. Hermaphroditism in teleost is very rare, and it may be synchronous where the gonads contain both male and female gametes or may

develop as sequential form, which exists as either protogynous or protandrous type. Protogynous develop into a female but later transforms into male, producing spermatozoa. However, protandry initially develops into a male but later switch to female, producing spawning eggs (Avise and Mank, 2009). Most of the teleost species adopt a variety of reproductive strategies such as variation in the reproductive modes, external fertilization, biparental caring, body growth, and small egg size to obtain high fecundity and reproductive success (Andersen *et al*., 2008). However, reproductive strategies undergo modifications based on some environmental or social conditions.

1.2 Influence of environmental factors on fish reproduction

Reproduction in fish is influenced by several environmental factors such as water flow, water quality, temperature, photoperiod, salinity, monsoon, and other climatic conditions. The environmental cues can be interpreted by fish species, which then synchronize with internal endogenous cycles for favorable reproductive conditions thereby results in successful breeding, and better survival of offspring (Ocasio-Torres *et al*., 2021). Water flow regimes directly or indirectly affects fish productivity as the alterations in the natural water flow by anthropogenic activities such as dam constructions, hydroelectric facilities, agriculture, industries, recreation, etc., changes various physical, chemical, and biological attributes leading to a decline in the water quality (Poff and Zimmerman, 2010). The quality of water directly affects the fish stock in the natural environment and indirectly

influences fish reproduction as well as the survival of the fish population. Dissolved oxygen, suspended particles, pH, hardness, and alkalinity are the major water quality parameters that play a significant role in fish reproduction (Viadero, 2019).

Water temperature has direct impacts on fish growth, survival, feeding response, spawning, and reproductive fitness. Optimum temperature varies among the fish species, and its deviation makes them less tolerant to changes in water quality, and become more susceptible to pathogens. Temperature mediates the metabolism and growths of aerobic microorganisms that exert an oxygen demand on water thereby indirectly affect the fish production system (Bartolini *et al*., 2015). Photoperiod, the day length variation, plays a prime factor in determining the growth and reproduction of fish. In some fish species, the development and maturation of gonads depend on the day length of the photoperiod and strongly affects the timing of puberty and sexual maturation (Hansen *et al*., 2001).

Salinity is deemed to be an important factor in controlling many stages of development, growth, and reproduction of fish including egg fertilization, yolk sac resorption, early embryogenesis, inflation of swimbladder, and larval growth (Boeuf and Payan, 2001). The monsoon with heavy rainfall, less water conductivity, and high water level plays a significant role in preparing breeding grounds for reproduction, and also initiates gonadal maturation in fishes of the tropical regions (Sreekanth *et al*., 2016). Environmental factors may be either ultimate or proximate factors, which influence the temporal pattern of reproduction in fishes (Lowerre-Barbieri *et al*., 2011). The

ultimate factors promote reproduction within specific and restricted time frames concerning the environment, while the proximate factors or continuing factors affect the timing of various physiological processes of reproduction such as stimulation of vitellogenesis, final maturation, ovulation, and oviposition (Wang *et al*., 2010). In some cases, proximate factors like adverse weather, insufficient food, predators, or pathogens may hamper the process of reproduction, changing the focus on taking refuge, shifting the habitats, or inducing an immune response (Wingfield, 2008).

1.3 Seasonality of reproduction

The seasonal changes are relatively less in tropical areas than the temperate regions. Seasonal reproduction is observed in fishes that inhabit both temperate and tropical areas, which is controlled by several intrinsic and extrinsic factors. The availability of food, temperature, rainfall, light, suitable substratum, area free from predators is the major extrinsic factors that influence seasonal reproduction. In addition, some of the intrinsic factors such as storage of fat reserves, duration and phase of the reproductive cycle, and mating behavior control fish reproduction (Bailey *et al.*, 2018).

The tropics are the region on the Earth that lies between latitudes 23.44ºN and 23.44ºS, where the sun directly overhead at least once a year. The areas between 5ºS and about 15ºN tend to have two rainy seasons in a year, each lasting approximately for two months. The areas away from the equator usually possess a single rainy season and a single dry season (Munro, 1990). The seasonal changes in

rainfall have two possible roles in the reproduction of fishes, namely by maximizing the individual fitness of reproduction, and by affecting the timing of reproduction (Wang *et al*., 2010).

Nutrients, feeding preferences, and feeding behavior are known to link together either to enhance or inhibit fish reproduction to some extent. Like other higher vertebrates, the increase in food consumption helps to store the energy as fat reserves in the body that determines the seasonality of the reproductive period in fish (Lall and Tibbetts, 2009). The potential force that limits the breeding seasons in fish is the interspecific competition for food among the juveniles and competition for spawning sites among the adults. The selection of different breeding seasons helps to reduce the competition among fishes, which is known to be caused by the overlapping of food resources (Kramer, 1978). The lunar cycle also influences spawning in fish where the young ones emerge in the night-light of the full moon to enhance parental protection from nocturnal predators (Desjardins *et al*., 2011).

Some of the endogenous stressors, influenced by extrinsic or intrinsic factors, reallocate the resources available for reproduction thereby contributes to their immediate survival than in the involvement of reproductive functions. The adverse effects of the stressors include failure of maturation, reduced gonadal mass, the decline in the levels of gonadotropins and gonadal steroids, low vitellogenin production, oocyte reduction, and increased frequency of oocyte atresia in the ovary (Leatherland *et al*., 2010). The social environment also leads to the disruption of reproduction as it was found in the aquaculture field that the dominant adults may sometimes inhibit the reproductive ability

of their subordinates (Jones and Thompson, 1980). Besides, some of the environmental stressors in the form of pollutants that functions as the endocrine disruptors also influence gamete maturation, development of secondary sex characters, spawning, and the mating of the fish. Thus the endogenous events are synchronized with the seasonally dependable environmental cues associated with seasonal, climatic events, and temperature cycles to ensure better survival and productivity.

1.4 Reproduction in teleosts

Reproduction is the biological process that ensures the transfer of genetic materials from generation to generation. The teleost fishes adopt different reproductive strategies and based on the season it may be seasonal breeders or annual breeders. Most of the teleosts are seasonal breeders as they perceive environmental cues, and they prefer to breed only during the most favorable season, while a few species are annual breeders, and they breed throughout the year (Juntti and Fernald, 2016). A wide range of freshwater teleosts is iteroparous, where they undergo reproduction continuously throughout their life, and also gonochoristic with distinct sex having external fertilization. However, some species are semelparous, where they spawn only once in their lifetime.

The morphological and histological studies in teleost gonads have documented cyclic variations during gonadal development. The gonadal variations are characterized by distinct phases including germ cell renewal, differentiation, development, maturation, and release of

gametes. These variations are associated with different ecological and behavioral adaptations during reproduction (Coward *et al*., 2002). Fish gonads are elongated, a paired organ situated laterally in the abdominal cavity, ventral to the air bladder, and dorsal to the alimentary canal (Melo *et al*., 2011). Generally, the right and left gonads of both sexes are united caudally to form a common duct that opens to the exterior through the anal opening (Martins *et al*., 2010).

1.4.1 Gonads and reproductive indices

Gonads of teleosts lack medullary tissues where the gonadal differentiation is more apparent in females than males. Ovary, the female reproductive organ, is a hollow paired structure that remains either fused in the middle or separated. The fish ovaries may be of gymnovarian or cystovarian types, where the former is characterized by the direct release of oocytes into the coelomic cavity, which then travels through the ostium, enters the oviduct, and get released. In the cystovarian type, the ova are directly deposited to the exterior through the oviduct (Urbatzka *et al*., 2011). The ovary consists of tunica albuginea lined by the connective tissues, smooth muscles, and blood vessels. The outer surface of tunica albuginea develops finger-like projections called ovigerous folds or ovigerous lamellae (Ganguly, 2013).

The teleost fishes exhibit three patterns of oocyte development namely synchronous, group-synchronous, and asynchronous (de Vlaming, 1983). The synchronous pattern is associated with semelparous species while the group-synchronous pattern is related to

the seasonal breeders, consisting of both immature and mature oocytes. The asynchronous pattern is found in multiple spawners enjoying a prolonged spawning season, consisting of all developing stages of oocytes (Wallace and Selman, 1981). Under the microscope, ovaries are seen in different stages of oocyte development namely chromatin nucleolus, perinucleolus, previtellogenic, vitellogenic, and atretic oocytes (Viana *et al*., 2018).

The testes of teleosts are elongated, paired organs attached to the dorsal body wall, and its structure varies from species to species. Basically, it exists in two forms namely lobular and tubular, where the former is the most typical of all teleosts species composed of numerous lobules separated by a thin connective tissue while the tubular type is restricted to the Atheriniform group. Tunica albuginea, the tough external membranous covering of testis, separates the tubular compartment or seminiferous tubules from the interstitial compartments (Grier, 1981). The seminiferous tubules are formed of two types of cells namely germ cells and somatic Sertoli cells. The interstitial compartment forms the testicular interstitium, composed of fibers, blood vessels, immune cells, and Leydig cells.

Depending on the species and the nature of spawning, different stages of spermatogenesis are identified as spermatogonia, spermatocyte, spermatid, and spermatozoa where their composition determines the stages of testicular maturity (Joshi and Joshi, 1989; Rutaisire *et al*., 2003). Fully matured and ripened gonads expel their eggs or mature spermatozoa into the surrounding aquatic medium, which aids in external fertilization. The entire period of attaining

maturity and spawning is referred to as the intense breeding season of the fish that are influenced by several internal and external factors (Pankhurst and Munday, 2011).

The physiological condition of the fish in the natural environment are determined using some biometric parameters including length-weight measurement (Onsoy *et al*., 2011), gonadosomatic index (Zeyl *et al*., 2014), hepatosomatic index (Hismayasari *et al*., 2015), levels of sex steroid hormones (Pham and Nguyen, 2019), and fecundity rate (Brewer *et al*., 2008). The length-weight relationship is a condition factor used to determine the biological changes in fish. The variation in the estimates of relationship occurs by the influence of several factors including sex, season, habitat, diet, stomach fullness, gonad maturity, health, and preservation techniques (Famoofo and Abdul, 2020). GSI is a widely accepted indicator to determine different stages of gonadal maturation (Hismayasari *et al*., 2015). The hepatosomatic index is used as an indicator of energy reserves where the energy stored in the body is utilized for reproduction (Chellappa *et al*., 1995). Vitellogenin production causes variations in HSI during the reproductive cycle, mainly in females, whereas such variations are less expressive in males (Reading *et al*., 2017).

Fecundity is a measure of reproductive potential and an important ecological parameter displaying the population dynamics of a species. It is an important factor in fish stock management where it quantifies the reproductive capacity of individual fish by measuring the number of vitellogenic oocytes or mature eggs present in the ovaries,

immediately before spawning (Bagenal and Braum, 1978; Payne and Collinson, 1983). The major factors affecting the fecundity are environmental variations, availability of food, duration of the breeding season, spawning frequencies, age, and size of fish. The species that produces free eggs have high fecundity to compensate for its loss during the dispersion of eggs into inappropriate sites. However, species producing adhesive eggs or exhibiting parental care have low fecundity due to their less dispersion (Nikolsky, 1963). The fecundity rate also varies among the multiple spawners as it depends on different environmental conditions throughout the year (El-Sayed, 2020).

1.4.2 Regulation of reproduction

In fishes, the major reproductive events like gonadal development, maturation, ovulation, and spawning are controlled by the hypothalamus-pituitary-gonadal (HPG) axis. Gonadal steroid hormones play a prominent role in sexual differentiation, gonadal maturation, and reproduction. Besides, the sex steroid hormones directly influence the hepatic vitellogenin synthesis, a deposit of vitellogenin in the developing oocytes, gonadal development, maturation, and sexual behavior in female fish (Yaron *et al*., 2003; Nagahama and Yamashita, 2008).

The hypothalamus produces gonadotropin-releasing hormone, which acts on the pituitary gland for the synthesis and release of gonadotropins namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The gonadotropic hormones in turn act on the gonads for the secretion of sex steroid hormones for gonadal

development. The main hormones that are involved in reproduction involve estradiol and testosterone, the female and male sex hormones, respectively. The sex steroid hormones undergo a feedback mechanism by acting on the HPG axis to regulate the reproductive cycle (Yaron *et al*., 2003; Zohar *et al*., 2010). Besides, the role of kisspeptin and gonadotropin-inhibitory hormone plays an important role in the reproductive system, which stimulates and inhibits the neurons of the gonadotropin-releasing hormone, respectively (Shahjahan *et al*., 2014).

1.5 Relevance of the study

Reproductive biology is an important aspect of fisheries research as it guides in aquaculture, stock assessment, and management. The climbing perch, *Anabas testudineus*, an obligatory air-breathing species were selected in the present study to assess the reproductive cycle and gonadal development at first maturation in captivity under laboratory conditions. The breeding of the species in captivity practiced in India remains scanty, and the data on the reproductive biology of *A. testudineus* is relatively insufficient. The study supports the best practices for the species culture and survival since the fish has long been used as a food source in India. Besides, the study on the reproduction of the fish enables the evaluation of the risk assessment concerning pollution, stress, or climate changes. It is expected that the study provides the fundamental data necessary for fisheries management to strengthen the challenges faced due to overexploitation of the stocks, and failure of management.

2. OBJECTIVE OF THE STUDY

- To identify the species using molecular phylogeny and to assess the gonadal developmental stages in *Anabas testudineus*

3. REVIEW OF LITERATURE

Teleost fish exhibit diverse mechanisms to maintain successful reproduction in a challenging environment. The unique behavioral, anatomical, and physiological adaptations allow the fish species to adjust efficiently in the different ecological conditions. Several environmental changes such as land transformations, species invasions, agricultural practices, over-fishing, global climate changes, and pollution modify the reproductive strategies of the fish globally. Thus conservation of native fish species is important by enabling an awareness of habitat conservation, developing new aquaculture practices, and seed restoration through artificial propagation techniques. The knowledge of the reproductive biology of fish is critical for the proper stock management practices, development of selective broodstock, genetic improvement, and species conservation (Anupama *et al*., 2019).

Recently, several studies have paid much attention concerning the reproductive biology of fish to protect and conserve the inland fish species. *Anabas testudineus,* one of the indigenous fish species is facing challenges for its existence due to the changes in various ecological factors, including pollution in the inland water bodies thereby declines the seed production, culture, and farming (Rajan, 2015). Therefore, a team of aquaculturists across the world has collectively assessed the reproductive biology of *A. testudineus* to improve the reproductive potential and stock management, which is being reviewed in detail in this section.

The external morphology of *A. testudineus* has been studied in detail, and identified them as bisexual fish, but lack sexual dimorphism (Dehadrai *et al*., 1973; Banerjee and Prasad, 1974). However, sexual dimorphism has been identified based on the size and color of the body, bulginess of the belly, vent position, and fin coloration during the breeding season (Mookerjee and Mazumdar l946; Lagler *et al*., 1977; Behera *et al*., 2015a). *A. testudineus* is a slow growing fish that grows a maximum of up to 22 to 25 cm size under the laboratory condition (Hora and Pillay, 1962), while in the natural habitat of Kuttanad lake, Kerala, it reached only up to 18 to 20 cm long (Kumary and Raj, 2016). The attainment of first sexual maturity in both males and females occurs at approximately 8 cm in length or within about six months of age (Chanchal *et al*., 1978; Ndobe *et al*., 2020).

The fecundity of *A. testudineus* of 12.3-15.5 cm length and 30.24-66.62 g weight has been found to vary from 12,355 to 41,820 eggs (Banu *et al*., 1985). The gonadal development and rise in gonadosomatic index (GSI) occurs usually during May, where females mature earlier than the males showing an increase in ova diameter as the indication for the arrival of the spawning period (Banu *et al*., 1987). The gonadosomatic index and ova diameter has been used as an index for the identification of gonadal maturity and spawning period (Shashi and Akela, 1996). The juveniles are active feeders than the adults as evidenced by an increase in the length-weight relationship where the slow growth rate in adults had been attributed to the depletion of body reserves during gonad maturation. The maximum feeding intensity of

adults has been observed from October to December, or during the post-spawning phase (Dey *et al*., 2005).

Based on the GSI values and histology, the ovarian maturity of *A. testudineus* has been classified into five stages namely immature, maturing virgins, recovering spent, mature, ripe, and spent. Induced breeding of *A. testudineus* using commercial hormones under captivity has been shown to improve GSI and fecundity, and enhanced the success rate of fertilization and hatching (Sarkar *et al*., 2005). A study on the reproductive biology of *A. testudineus* has shown that 19.5 as the high gonadosomatic index with an egg diameter of 654.10 µm, and sperm-head length of 3.18 µm while the mean fecundity rate was 5,53,708 per kg body weight having an average of 4,480 eggs per g ovary weight (Hasan *et al*., 2007). Monoculture of *A. testudineus* in low stock density yielded a higher growth rate while the culture of *A. testudineus* with other species resulted in a low rate of larval survival thereby limiting the seed production (Kohinoor *et al*., 2007).

The rate of fecundity in *A. testudineus* of size 12.4-19.2 cm, and 33.22-137.19 g has been shown to vary from 3,120 to 84,690 with egg size of 0.54 to 0.80 mm diameter (Marimuthu *et al*., 2009). Similarly, another study also has detected a high fecundity rate varying from 2,430 to 41,600 eggs from the females of 9.8-14.2 cm, and 24.4-81.38 g weight. The study has also observed that the fish showed an intermittent spawning behavior, and the spawned eggs were in pelagic form (Zworykin, 2012). The high rate of fecundity observed in the studies under the controlled laboratory conditions suggested that the eggs did not require any parental care in the aquarium, where the

parent neither build a nest nor prepare a spawning substrate. The adults engaged in polygamy or promiscuity with more than one partner letting more number of spawning events or sequential spawning patterns during the breeding season (Zworykin, 2012).

In a culture system, it has been found that an increase in the depth of water decreased the stocking density while the optimal feed enhanced the rate of larval survival (Behera, 2013). The growth of *A. testudineus* has occurred at a faster rate in the natural pond culture than those cultured in the tanks and cages (Kumar *et al*., 2013; Ali *et al*., 2015). Besides, some studies have documented that *A. testudineus* supplied with probiotic food supplements such as *Bacillus licheniformis* enhanced the growth and hematological profiles along with the resistance to diseases thereby increased productivity (Kumari *et al*., 2013; Sumon *et al*., 2015; Anantharaja *et al*., 2017).

The induced breeding of *A. testudineus* using the synthetic gonadotropin-releasing hormone has revealed an increased spawning success with a high fecundity rate holding 1: 2 as the female to male ratio (Mandal *et al*., 2016). In another study, an injection of synthetic hormone WOVA-FH to *A. testudineus* has been shown to stimulate the male courtship behaviors such as pairing and chasing of the female thereby resulted in breeding success with a high rate of spawning, fecundity, fertilization, and hatching with an increase in male to female ratios (Behera *et al*., 2016). The breeding season of the Vietnam strain has been identified in May showing high GSI values in both sexes, and the fecundity ranged from 50,610 to 2,27,378 eggs, which was proportional to the total length, body weight, ovary weight, and ova

diameter (Hafijunnahar *et al*., 2016). In a comparison study, it has reported that the growth and reproduction potential was high for the Vietnamese strain than the Thai strain (Kohinoor *et al*., 2016). However, the cultured Thai Koi has been enriched with nutrients like lipid, protein, fiber, and carbohydrates (Mondal *et al*., 2019).

A study has evidenced that F1 and F2 broodstocks of *A. testudineus* supplemented with vitamin E diet showed better reproductive performance in the second generation female broodstock than F1 generation as evidenced by an increase in growth rate, fecundity, and egg diameter, while the broodstocks supplemented with vitamin C and *Spirulina sp*. showed less reproductive performance (Helmizuryani *et al*., 2018). Another study has reported that the fish supplemented with the probiotics-containing *Bacillus subtilis* and *Aerobacter sp*. resulted in the highest growth in terms of net length and weight gain along with the highest value of GSI than the fish supplemented with vitamin C in the diet (Hossain *et al*., 2020).

The study of length-weight relationship has identified that male *A. testudineus* tend to be smaller than females where the male with mature testis was noted at 6.2 cm length and 4.1 g weight whereas mature ovary in female occurs only it attains the size of 7.2 cm and 6.8 g. Further, the gravimetric estimation has observed that the fecundity varied from 1,556 to 70,973 in females attaining the peak spawning period during July-August (Ndobe *et al*., 2020). Supplementation of ovatide, a synthetic hormone, has been shown to induce better reproductive performance in *A. testudineus* as evidenced by a high percentage of fertilization, hatching, and survival rate, and

recommended as the more effective spawning inducer for mass seed production (Rajbongshi *et al*., 2020). In a cross-breeding study conducted in the hatcheries of Bangladesh, it has been shown to modulate the reproductive outcomes, growth performances, and muscular growth of *A. testudineus.* This was evident by the gradual increase in pre-spawning season and decrease in post-spawning season with the highest value of GSI in April and lowest in July, thereby considered as a superior stock in the future breeding and stock improvement programme (Ahammad *et al*., 2021).

The overall review of the literature on the reproductive biology of *A. testudineus* has stated that the species has been well-explored in south east-Asian counties, especially in Bangladesh as well as in North India. However, the studies on the reproductive biology of the fish are relatively less in Kerala under the controlled laboratory condition. Hence the current study was established to understand the reproductive cycle and gonadal development of the fish, *A. testudineus* under captivity.

4. MATERIALS AND METHODS

4.1 Molecular identification of *Anabas testudineus* using mitochondrial Cytochrome Oxidase I (COI) gene

The molecular identification of fish specimens reared in the laboratory, which were collected from the fish farm Pulimugham Hatcheries, Alappuzha District, Kerala (10º 37' N; 76º 31' E) was compared with the specimens from the backwaters of river Chaliyar, Kozhikode district (11° 15' N; 75° 56' E). The information regarding the date of collection, locality, etc., about each specimen was recorded. Muscle tissues of fish collected from two sites were preserved in 70% ethyl alcohol for the extraction of whole DNA using Origin® DNA extraction kit following the manufacturer's instructions.

The extracted DNA was amplified for the Cytochrome Oxidase subunit I (COI) gene by Polymerase Chain Reaction (PCR) using the forward primer 5'-TCAACCAACCACAAAGACATTGGCAC-3' and reverse primer 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3' (Chakraborty and Ghosh, 2014). The PCR reaction was performed in a 50 µl reaction volume containing 5 µl of template DNA, 1 µl of 10 mM dNTPs, 0.5 µl Taq polymerase, 5 µl of reaction buffer, 1 µl of each primer, and nuclease-free water. The thermal profile of COI amplification was as follows: An initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 10 sec, annealing temperature at 50 °C for 1 min, and finally elongation at 72 °C for 45 sec followed by a hold at 4 °C. The obtained PCR-amplified products were analyzed using 1% agarose gel electrophoresis containing

ethidium bromide stain. The single uniform band was then purified using Gel extraction kit (Qiagen, USA), following the manufacturer's instructions.

The amplicons were bidirectionally sequenced using an automated sequencer ABI 3730XL by Sangers method (Sanger and Coulson, 1975). The COI barcode sequences generated were blasted in the NCBI-BLAST tool (www.ncbi.gov) and similar sequences in the NCBI database were retrieved for further sequences analysis (Altschul *et al*., 1990; Benson *et al*., 2006). The retrieved sequences and sequences of each species generated from this study were compared, and aligned using Clustal W in BioEdit software to ensure the sequences are clear without any mismatches. The phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA-7.0 software), 7.0.9 beta version, using multiple aligned sequences of partial Cytochrome Oxidase I gene by the Neighbour-joining method. The reliability of the Maximum likelihood phylogenetic tree was estimated using bootstrap values run for 1000 replicates (Kumar *et al*., 2016).

4.2 Housing of the test animal

After molecular identification, fingerlings of *Anabas testudineus* (2 ± 0.6 cm, approximately 2 months old) collected from the Pulimugham Hatcheries, Alappuzha District, Kerala, India were reared in the laboratory conditions until it attains maturity i.e., at 7 ± 1 g; 7 ± 1.5 cm (Jacob, 2005). Fish were fed twice daily with the

standard protein pelleted feed (3% of the body weight) that contained 28% crude protein, 3% crude fat, 4% crude fiber, and 10% moisture.

4.3 Ethics statement

Experiments conducted using *Anabas testudineus* as the test model strictly followed the guidelines approved by the relevant authorities in India namely the Animal Welfare Board of India, and Committee for Control and Supervision of Experiments on Animals (CPCSEA) under the Ministry of Environment, Forest, and Climate change, Government of India. All the experimental protocols performed in the thesis work were approved by the Departmental Research Committee of the Department of Zoology, University of Calicut, Kerala, India.

4.4 Standardization of water quality parameters

The water quality parameters were monitored using the APHA guidelines (APHA, 1998). The water temperature (28 ± 2 °C), salinity (<100 ppm), pH (7.4-7.6), and dissolved oxygen (8.64 ± 0.6 mg L^{-1}) were monitored and maintained throughout the study. Other physico-chemical parameters including alkalinity and hardness were measured by analyzing the concentrations of particulate matter, unionized ammonia, nitrate, chlorine, total organic carbon, and other metallic impurities, which were found within the normal range was retained throughout the study period to ensure the best fit for the survival and growth of the fish.

4.5 Test parameters

Fish were randomly selected from the stock maintained in the laboratory, and morphology was noted prior to the experiment. The length and weight of the fish collected in every month were recorded to the nearest unit, and represented in different stages of gonadal development. Gonads and liver were dissected out and weighed accurately at every month to calculate the respective relative weights of gonadal development. The relative weight of gonads (testes and ovary) or gonadosomatic index (GSI), and the relative weight of liver tissues (from male and female fish) or hepatosomatic index (HSI) were evaluated using the standard formula, and expressed in percentage (King, 1995; Sulistyo *et al*., 2000).

GSI or HSI = (Tissue weight (g)/ Fish weight (g)) × 100.

The fecundity was estimated by the gravimetric method using the ovaries from spawning stage. Briefly, after 48 h of preservation in modified Gilson's fluid, the eggs were completely liberated, washed thoroughly, and spread on the blotting paper to air dry, and counted using the sub-sample by the given equation (Grimes and Huntsman, 1980).

$F = nG/ g$

Where, F = fecundity, n = number of eggs in the sub-sample, G = total weight of the ovaries, g = weight of the sub-sample. On the basis of the above parameters, gonadal developmental stages were classified into preparatory (PRE), pre-spawning (PRP), spawning (SP), and post-spawning (PSP) stage (Chakraborti and Bhattacharya, 1984; Pal *et al*., 2018).

4.6 Histological analysis

Gonadal tissues were dissected, rinsed in physiological saline to remove the tissue debris and clotted blood, and then fixed in 10% buffered formalin for 24-48 h. The tissues were dehydrated in ascending series of alcohol and cleared in xylene until they became translucent. The tissues were embedded in molten paraffin wax for an hour for the complete impregnation to make the tissue blocks. Sections were made using rotary microtome at 4 to 6 µm thickness, which was double stained with hematoxylin and eosin, and finally mounted in DPX mountant (Roberts and Smail, 2001). Briefly, triplicate slides of ovarian and testicular sections were examined under a light microscope at 40 or 400X magnifications using a canon shot camera fitted to the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope.

The percentage of oocyte composition was measured using the ratio of number of oocytes to the total number of oocytes in ten frames per stage (Bernal *et al*., 2015), and the diameter of the oocyte was presented in µm. The size of spermatogenic cells were measured using Image J analysis software, version 1.52a (National Institutes of Health, USA), and the values are expressed in µm. The composition of spermatogenic cells were analyzed as the area covered by each cells to the total area of the field, and the values are expressed in percentage (Bernal *et al*., 2015).

4.7 Serum hormone analysis

Blood collected from the caudal vein of male and female fish were transferred into non-heparinized tubes and kept undisturbed for

30 min. The supernatant was centrifuged at 2500 rpm for 10 min at 4 °C to obtain the serum, which was then stored at − 80 °C. Analysis of testosterone and estradiol hormones were done in the blood serum according to the prescribed protocols provided in the commercial ELISA kits purchased from Bioassay technology laboratory, China.

4.8 Statistical analyses

The statistical package SPSS V-21.0 was used as the statistical tool to analyze various morphometric and hormonal parameters. Levene's test was conducted to ensure the equality and homogeneity of variance. One-way analysis of variance (one-way ANOVA) followed by Duncan's multiple range as post-hoc test was performed to set the significance within and between the variables. The data were presented as mean ± standard deviation (SD) in graphs setting significance at $P < 0.05$, and was denoted by different superscripts against each data point. Correlation between the fecundity and fish weight, length, and weight of ovary were performed using Pearson's correlation coefficient.

5. RESULTS

5.1 Molecular identification of *Anabas testudineus* using mitochondrial cytochrome oxidase I (COI) gene

Barcode sequences of cytochrome oxidase I (COI) gene for *Anabas testudineus* collected from Alappuzha and Kozhikode districts, Kerala, India were obtained with sequences above 500 bp size. The analysis of sequence did not detect any stop codon or frameshifts indicating that there were no pseudogenes. Information from the National Centre for Biotechnological Information (NCBI) obtained by BLAST search found similar barcode sequences of *Anabas testudineus* matching with the species of GenBank. The codon structure of the COI gene obtained from the Alappuzha and Kozhikode districts was CT-biased. The amplified partial nucleotide sequences of the COI gene isolated from *A. testudineus* of Alappuzha site were Adenine 23.37%, Thymine 31.37%, Cytosine 28.76%, and Guanine 16.5% while the fish collected from Kozhikode site showed the nucleotide frequencies as Adenine 22.99%, Thymine 30.75%, Cytosine 29.28%, and Guanine 16.98%. The database obtained was deposited to the GenBank in the Accession Nos. MZ604324.1, MZ604339.1 (Alappuzha, Kerala), and MZ604336.1, MZ604341.1 (Kozhikode, Kerala).

The phylogenetic tree generated through the neighbor-joining method using K2P distance showed two major clades with high bootstrap values (Fig. 1). The two clades of the phylogram revealed a distinct separation of *A. testudineus* collected from the two different

sites. The fish collected from Alappuzha district (shown as Accession Nos. MZ604324.1 and MZ604339.1 in clade 2 of Fig. 1) showed closeness to *A. testudineus* reported from Vietnam, Bangladesh, and Malaysia. The fish collected from Kozhikode district (shown as Accession Nos. MZ604336.1 and MZ604341.1 in clade 1 of Fig. 1) showed sequence similarity with *A. testudineus* reported from Philippines, Indonesia, France and Kochi, Kerala. Besides, another subclade of *A. testudineus* reported from West Bengal, India shared a sister-clade relation with the species obtained from the Alappuzha district. The ability of the COI gene in distinguishing two different species was proved through the phylogram of different subclades segregated as *A. cobojius* reported from Kochi of Kerala, India, and Bangladesh.

5.2 Morphological identification of sex and gonadal stages

Morphology of *A. testudineus* observed during the period from February 2017 to January 2018 showed sexual distinction in three main features like body color, belly bulginess, and presence of vent. Fish were then randomly sampled from the stock, and the abdomen is carefully dissected for confirming the sex, and identified different developmental stages of gonads. The present study identified different stages of gonadal development as follows:

Stage I: Preparatory (PRE) stage - February and March,

Stage II: Pre-spawning (PRP) stage - April to June,

Stage III: Spawning (SP) stage - July and August

Stage IV: Post-spawning (PSP) stage - September to January

No sexual dimorphism was observed in the fish, however morphological changes like body color, bulginess in the belly, and color of vent were prominent during breeding season (Table 1; Fig. 2).

5.3 Length and weight of the fish

The average weight and length of the fish showed progressive increase from the preparatory to post-spawning period in both sexes (Figs. 3 and 4).

5.4 Absolute weights of tissues

The month-wise and reproductive stage-wise weights of liver and gonadal tissues in both sexes showed significant variations. The weights of liver tissues in female and male fish showed a remarkable increase during the late post-spawning to preparatory stage (Table 2). However, the weights of gonadal tissues showed a notable increase during pre-spawning and spawning stages of reproduction (Table 2).

5.5 Relative weights of tissues

5.5.1 Gonadosomatic index

The gonadosomatic index (GSI) showed the peak values from April to August in both sexes, which were identified as the pre-spawning and spawning stages of reproduction (Figs. 5-7).

5.5.2 Hepatosomatic index

The hepatosomatic index (HSI) showed remarkable increase from September to March in female and male fish, which were

identified as post-spawning and preparatory stages of reproduction (Fig. 5, 6, and 8).

5.6 Fecundity

The relationship of fecundity to the body weight (Fig. 9A), ovary weight (Fig. 9B), and fish length (Fig. 9C) showed a very high degree of positive correlation. The number of eggs produced ranged between 6,500-11,000 in a female parent weighing 7.5 to 9.5 g with the regression equation as $y = 1050.5x$; $R^2 = 0.8928$ and $r = +0.9528$ (Fig. 9A) possessing the ovary weight between 900 and 1800 mg that occur in the spawning period of reproductive phase showing the regression equation of $y = 6.2803x$; $R^2 = 0.6557$ and $r = +0.9353$ (Fig. 9B), and the fish size of 6.5 and 9 cm range with the regression equation of $y = 1093x$; $R^2 = 0.8848$ and $r = +0.9024$ (Fig. 9C).

5.7 Histomorphological analysis of ovary

Microscopic examination of the ovary revealed different developmental stages throughout the reproductive cycle. Both chromatin nucleolus and perinucleolus stages occur from February to March. Chromatin nucleolus consists of a single, large nucleus, while perinucleolus contains a thin band of dense, compact, and basophilic cytoplasm surrounding the nucleus having an irregular nuclear membrane and 6-12 peripheral nucleoli (Figs. 10A and 10B), representing a preparatory stage of ovary (Fig. 11A). The chromatin nucleolus of 35-37 µm diameter contribute 41.3%, and the perinucleolus stage of 73-76 µm diameter occupies 35.4% of the

preparatory ovary, and the remaining 23.3% were formed of previtellogenic, vitellogenic, mature, and atretic stages (Figs. 12 and 13).

In April to June, the previtellogenic ovary (166-174 µm diameter) begins to develop vitellogenic follicles filled by yolk granules and cortical alveoli possessing a centrally located germinal vesicle (Fig. 10C), which was identified as the pre-spawning stage of the ovary (Fig. 11B). The pre-spawning oocyte consists of 28.7% of previtellogenic and 35.3% of vitellogenic stages while 36% of the oocyte was formed of chromatin nucleolus, perinucleolus, mature and atretic stages (Figs. 12 and 13). Later from July to August, the eggs were matured to pale yellow color with centrally located large oil vacuole, and the loosely arranged spherical eggs occupy the ovary (Figs. 10D and 10E) and are recognized as the spawning stage of the ovary (Fig. 11C). In the spawning stage, 35.9% of vitellogenic (447-469 µm diameter) and 44.4% of mature (599-625 µm diameter) oocytes occupied the ovary followed by previtellogenic, perinucleolus, atretic, and chromatin nucleolus (Figs. 12 and 13). The post-spawning stage of the reproductive cycle occurs from September till January that are identified with a mixture of atretic, spent as well as some previtellogenic and immature oocytes (Figs. 10F and 10G; 11D). In this stage, chromatin nucleolus (43.2%) predominates followed by perinucleolus (29.3%) and atretic (20.7%) oocytes (Fig. 12).

5.8 Histomorphological analysis of testis

All spermatogenic stages of testis were present throughout the annual reproductive cycle (Fig. 14A). However, certain stages are found abundant in specific months; accordingly, the stages are identified as preparatory stages from February to March months having abundant spermatogonia, spermatocytes, spermatid, and relatively fewer spermatozoa (Fig. 14B). The pre-spawning stage was observed between April and June consisting of all stages of spermatogenic cells enclosed in a large seminiferous tubules (Fig. 14C). The spawning stage was identified from July to August known by the presence of abundant spermatozoa while the other spermatogenic cells were rare, and enclosed within the dilated seminiferous tubule (Fig. 14D), and milt was easily extruded upon exerting slight pressure on the belly. The post-spawning stage begins during the September to January month characterized by loosely packed spermatozoa while some part of the seminiferous tubule remains empty, and other spermatogenic cells begin to appear (Fig. 14E).

The composition and size of different stages of spermatogenic cells were also estimated in the male fish. It was found that the percentage of spermatozoa was 9.6 in the preparatory stage, while it increased to 82.05% and 93.9% in pre-spawning and spawning stages, respectively, however, decreased in the post-spawning stage to 41.1% (Fig. 15). The size of spermatogenic cells (µm) varied from 1.56±0.06, 0.80±0.04, 0.61±0.06, and 0.37±0.09 for spermatogonia, spermatocyte, spermatid, and spermatozoa, respectively (Fig. 16).

5.9 Gonadal hormones

In female fish, the level of serum estradiol showed a significant ($P<0.05$) increase during PRP and SP stages of ovarian development along with a slight increase in the level of testosterone when compared with PRE and PSP stages (Fig. 17). In male fish, there was a progressive and significant ($P<0.05$) increase in the level of testosterone from PRE to PRP stages during testicular development while a gradual decrease was observed thereafter with a significant ($P<0.05$) reduction in the PSP stage (Fig. 18). Similarly, the serum level of estradiol was decreased significantly ($P<0.05$) in PSP stages when compared with the other stages during the process of testicular development (Fig. 18).

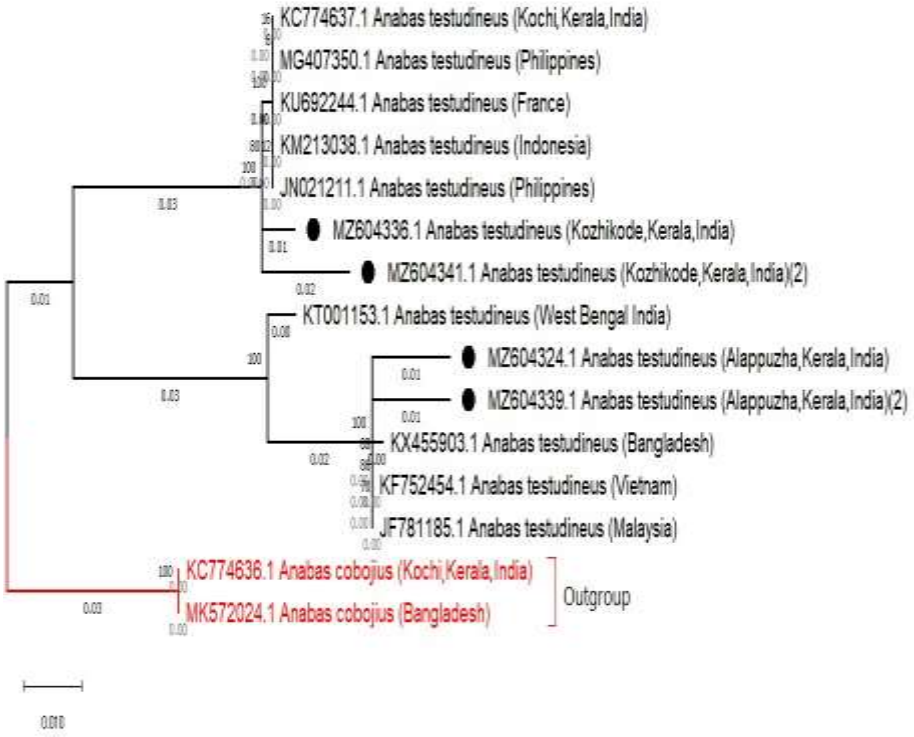


Fig. 1 Phylogenetic tree based on COI sequences of the freshwater fish *Anabas testudineus* collected from Kozhikode and Alappuzha districts. Species collected for the present study are bullet labeled.

Table 1 Morphological identification in *Anabas testudineus* during gonadal development

Month and Stages	Body color		Belly appearance		Color of vent		Color of gonad		Gonadal maturity	
	Female	Male	Female	Male	Female	Male	Ovary	Testes	Ovary	Testes
Feb-March (Preparatory)	Dorsal riffle green and ventral yellow		Not bulged		Reddish	Pale yellow	Pinkish or reddish brown	Yellow	Immature	Immature or maturing virgins and recovering spent
April-June (Pre-spawning)	Dark to pale yellow		Slightly bulged		Reddish		Yellow	Creamy white	Vitellogenic	Mature and ripe
July-Aug (Spawning)	Dorsal dark to pale yellow, and ventral reddish yellow		Bulged prominently	Slightly bulged	Reddish		Brownish yellow	Creamy white	Mature and ripe	
Sept-Jan (Post-spawning)	Dorsal riffle green and ventral pale yellow		Not bulged		Pale yellow		Brown	Reddish white	Spent or Rudimentary	

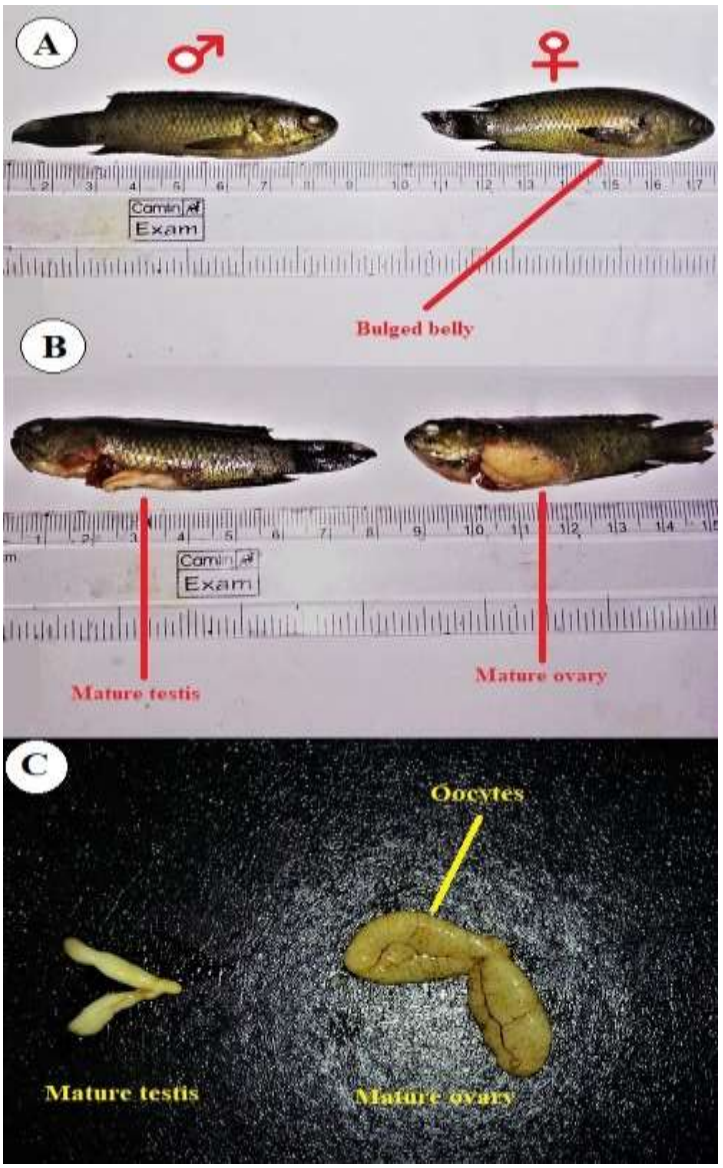


Fig. 2 **Male and female *Anabas testudineus* in the spawning season (A) Fish showing sexual dimorphism as bulged belly in the female (B) Exposed testis and ovary (C) Morphology of gonads showing mature bilobed testis and ovary with vitellogenic oocytes**

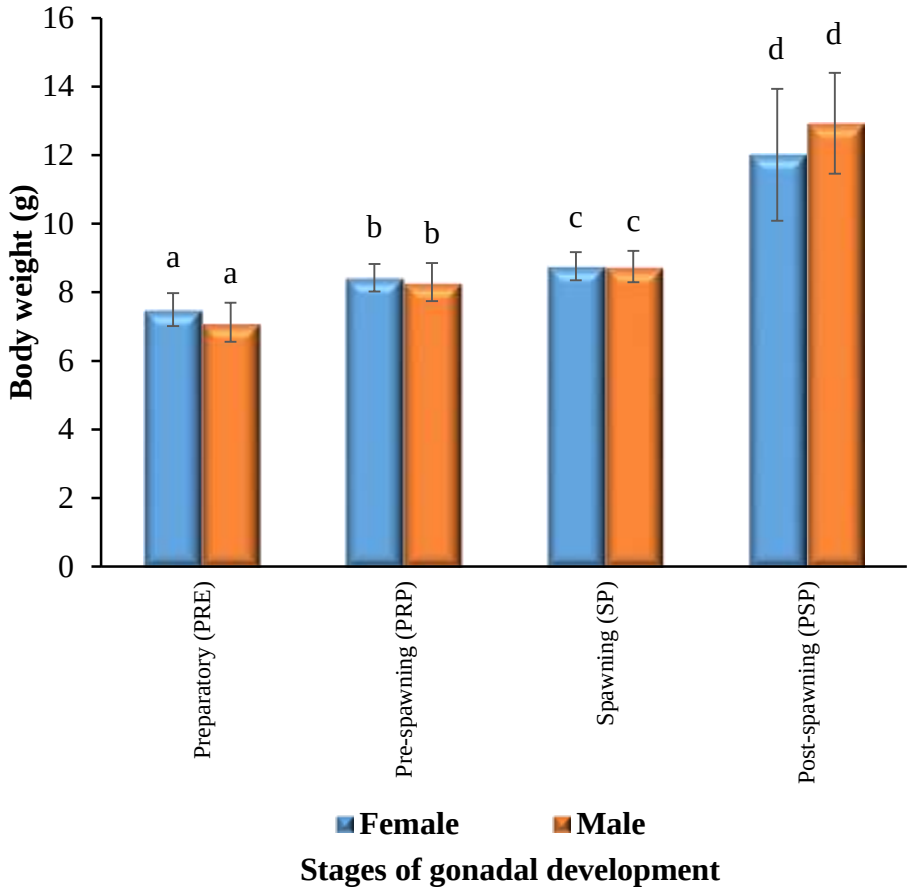


Fig. 3 Body weight of the fish *Anabas testudineus* during different stages of gonadal development (Mean ± SD; n = 30/ sex/ stage; Significant differences among means of each sex are indicated by different letters in superscripts, P<0.05)

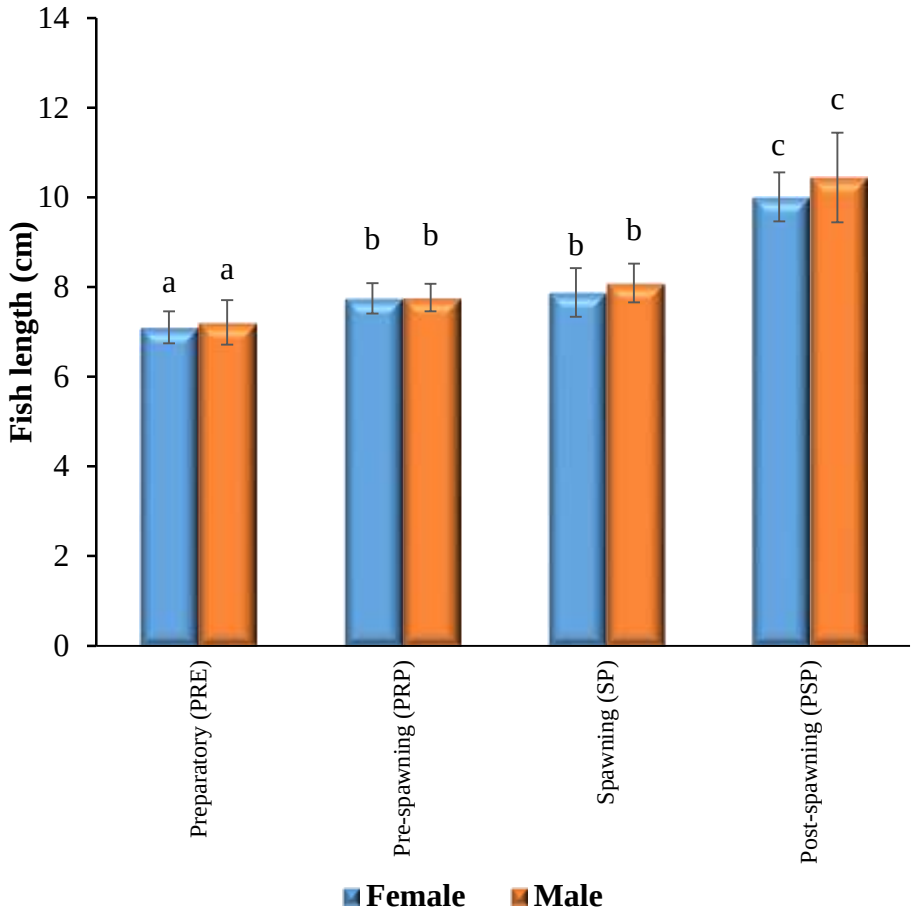


Fig. 4 **Length of the fish *Anabas testudineus* during different stages of gonadal development (Mean ± SD; n = 30/ sex/ stage; Significant differences among means of each sex are indicated by different letters in superscripts, P<0.05)**

Table 2 Variations in the tissue weights of the fish, *Anabas testudineus* during different stages of gonadal development (Mean ± SD; n = 30/ sex/ stage; Significant (P<0.05) differences among means of each sex are indicated by different letters in superscript)

Tissue weights (mg)	Preparatory (PRE)	Pre-spawning (PRP)	Spawning (SP)	Post-spawning (PSP)
Liver - Female (mg)	118.61±0.05[a]	74.19±0.15[b]	73.36±0.64[c]	83.36±0.74[d]
Liver - Male (mg)	97.07±0.05[a]	83.12±0.05[b]	81.50±0.23[c]	87.50±0.14[d]
Ovary (mg)	438.4±12.9[a]	926.9±11.8[b]	1205±13.9[c]	86.53±2.21[d]
Testis (mg)	45.50±0.94[a]	90.39±3.39[b]	120.7±3.17[c]	22.05±0.96[d]

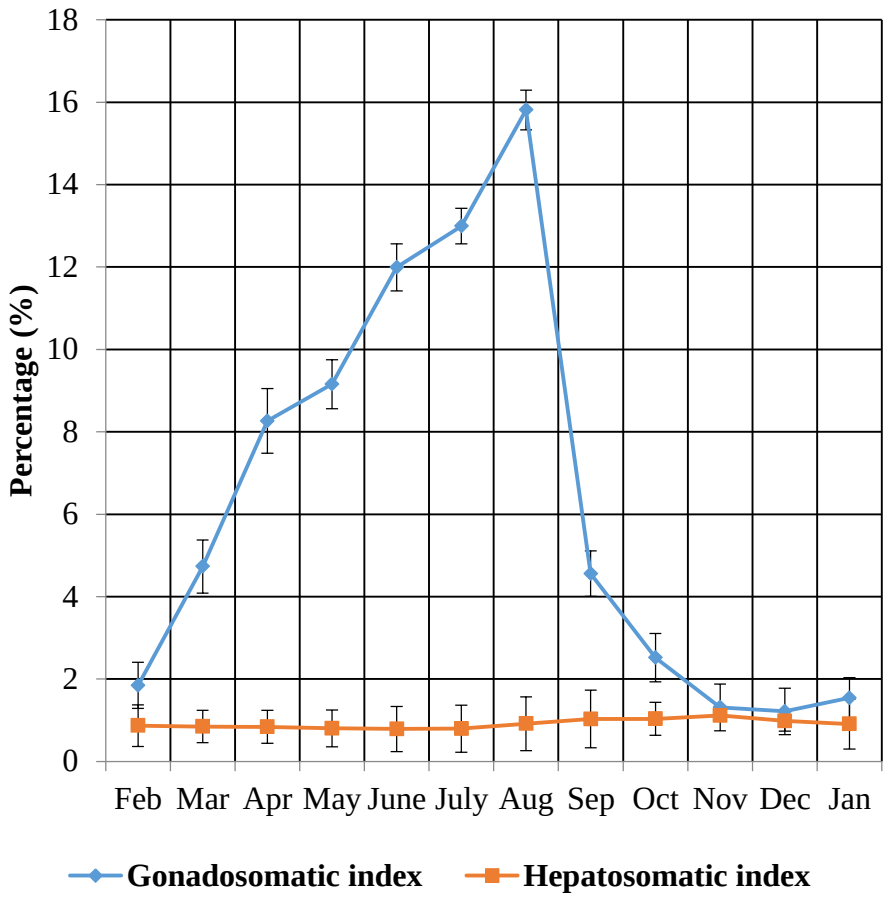


Fig. 5 Monthly variations in gonadosomatic index and hepatosomatic index in the female fish, *Anabas testudineus* (Mean ± SD; n = 10/ sex)

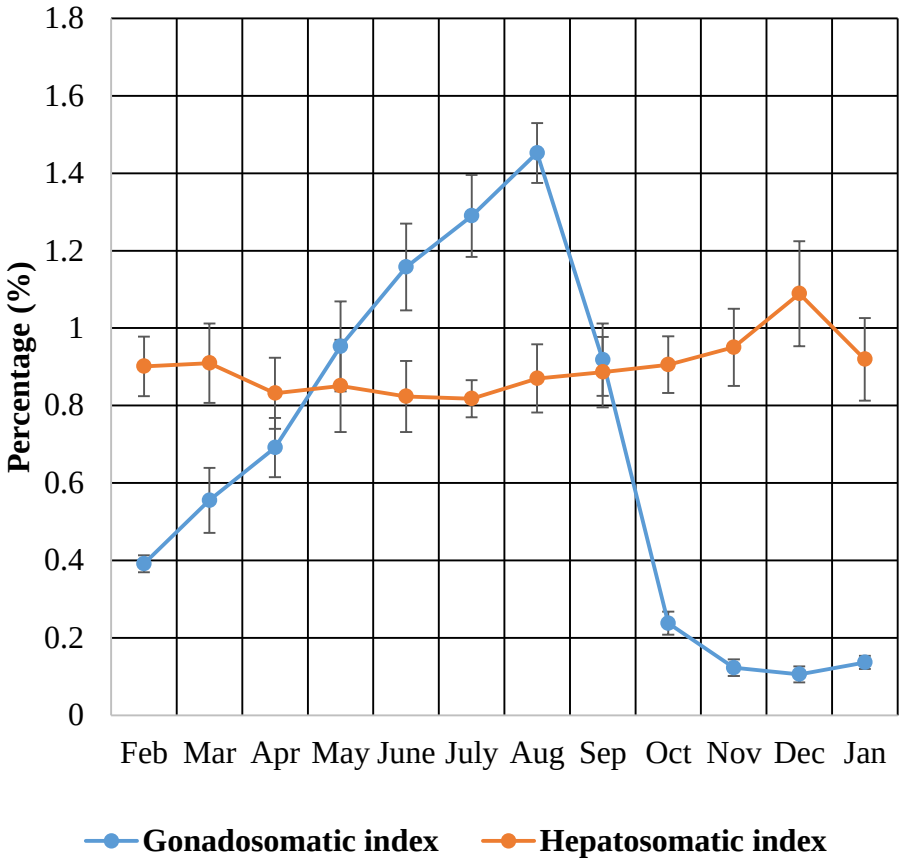


Fig. 6 Monthly variations in gonadosomatic index and hepatosomatic index of the male fish, *Anabas testudineus* (Mean ± SD; n = 10/ sex)

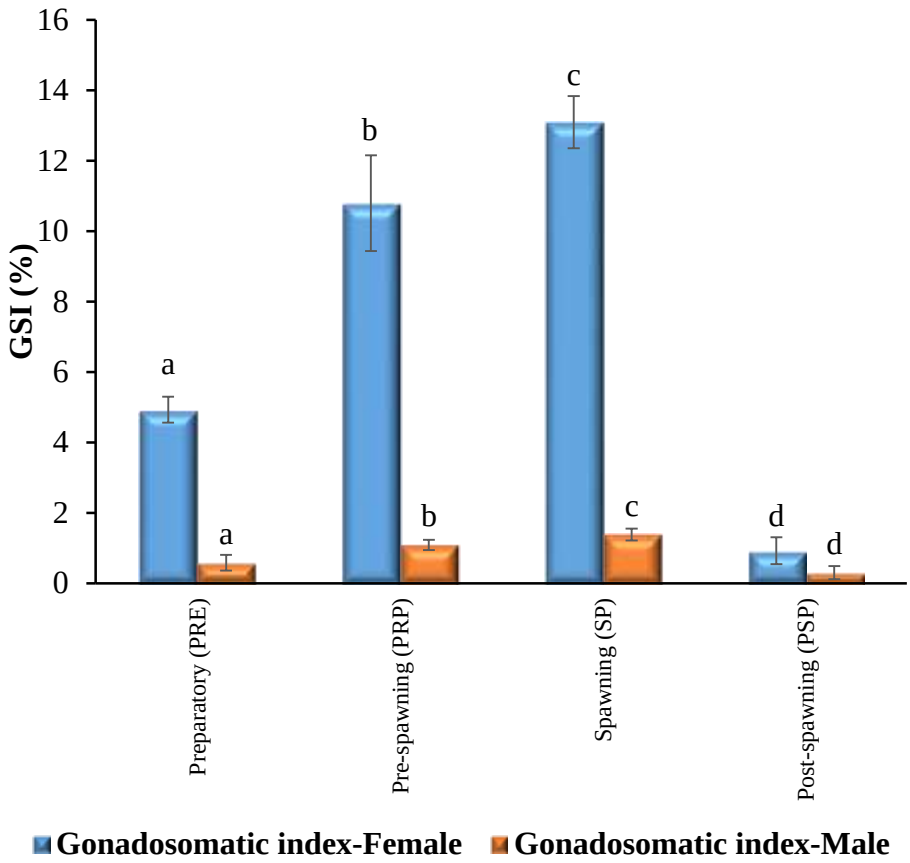


Fig. 7 Gonadosomatic index of the fish, *Anabas testudineus* during different stages of gonadal development (Mean ± SD; n = 30/ sex/ stage; Significant (P<0.05) differences among means are indicated by different letters in superscript)

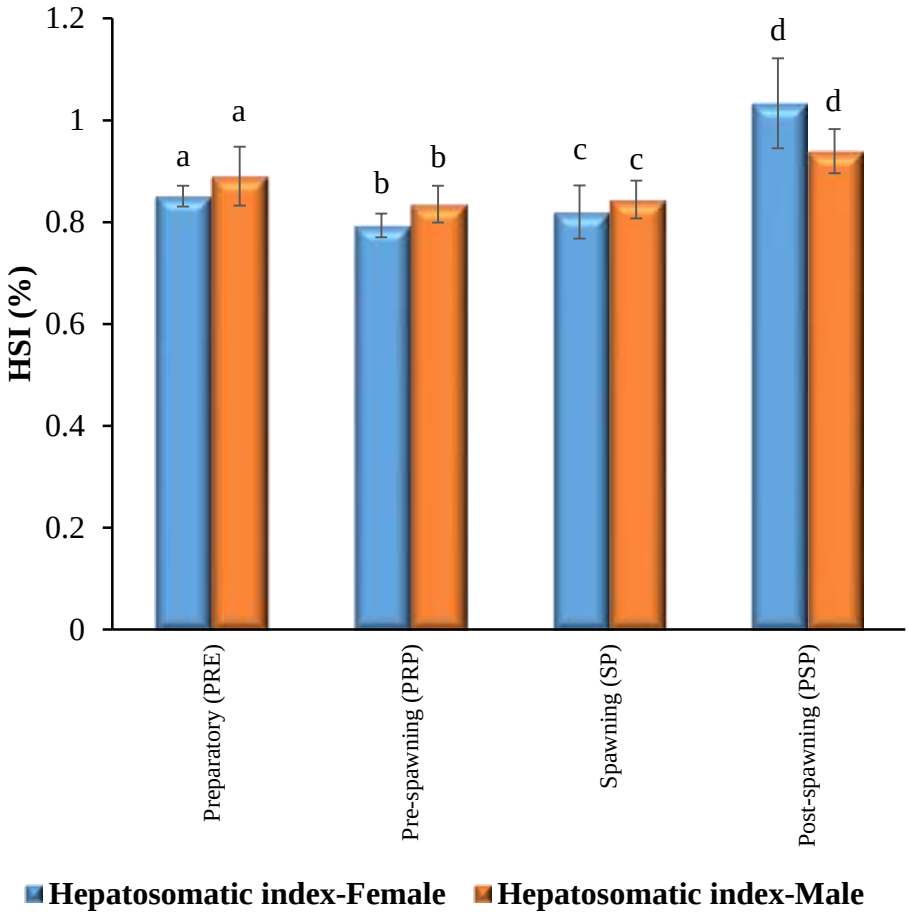


Fig. 8 Hepatosomatic index of the fish, *Anabas testudineus* during different stages of stages of gonadal development (Mean ± SD; n = 30/ sex/ stage; Significant (P<0.05) differences among means in each sex are indicated by different letters in superscript)

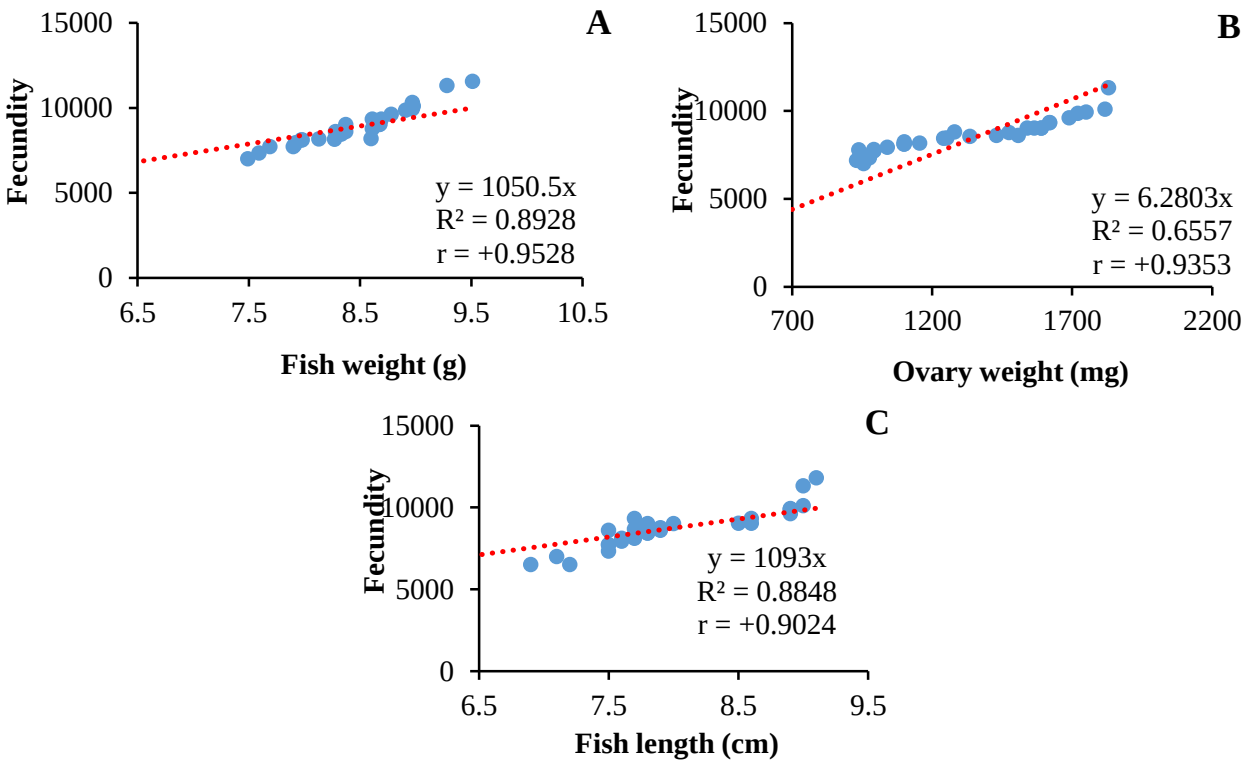


Fig. 9 Correlation between fecundity and fish weight (A), ovary weight (B), and fish length (C) (n = 30)

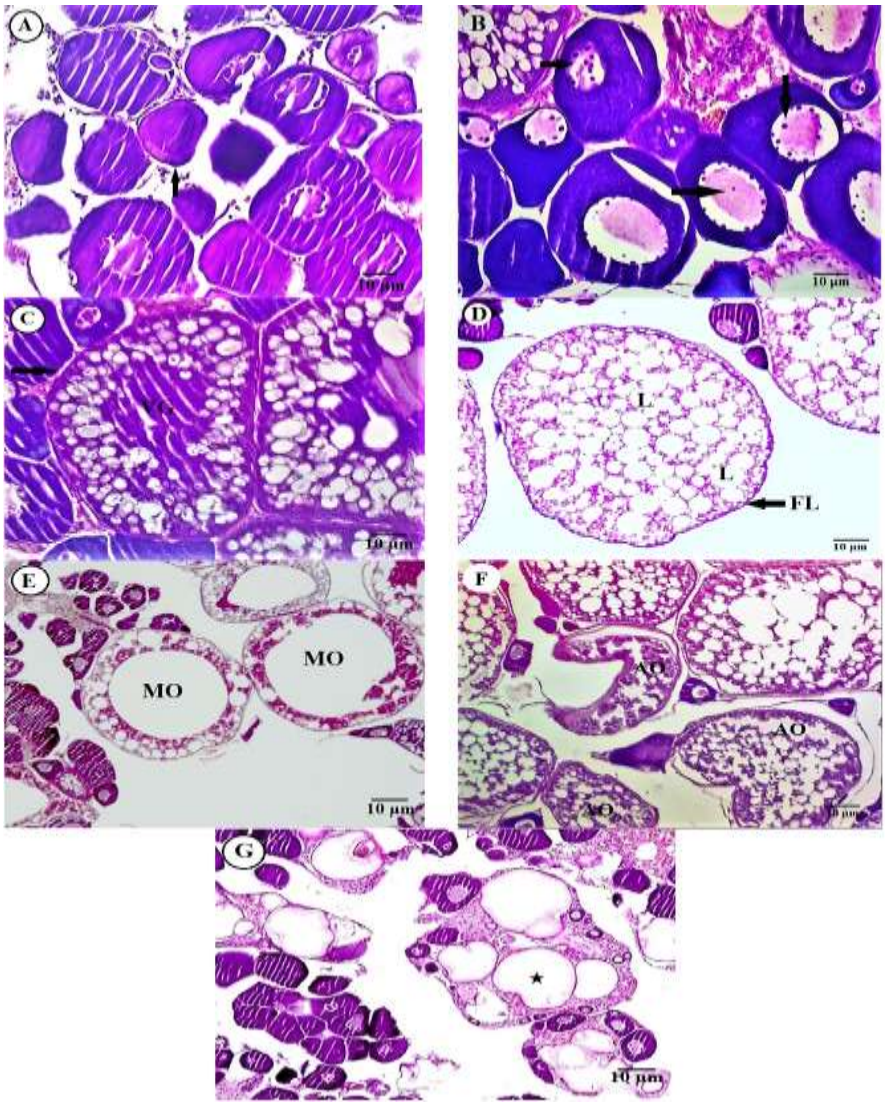


Fig. 10 Photomicrographs of oocytes in *Anabas testudineus* during different stages of gonadal development (A) Chromatin nucleolus stage (arrow), (B) Perinucleolus stage (arrow), (C) Previtellogenic stage; Follicular layer (Solid arrow); ca = cortical alveolus; YG-Yolk granule, (D) Vitellogenic stage; FL- Follicular layer; L-Lipid droplet, (E) Mature stage; MO-Mature oocyte, (F) Atretic stage; AO-Atretic oocyte, (G) Spent stage (asterisks)

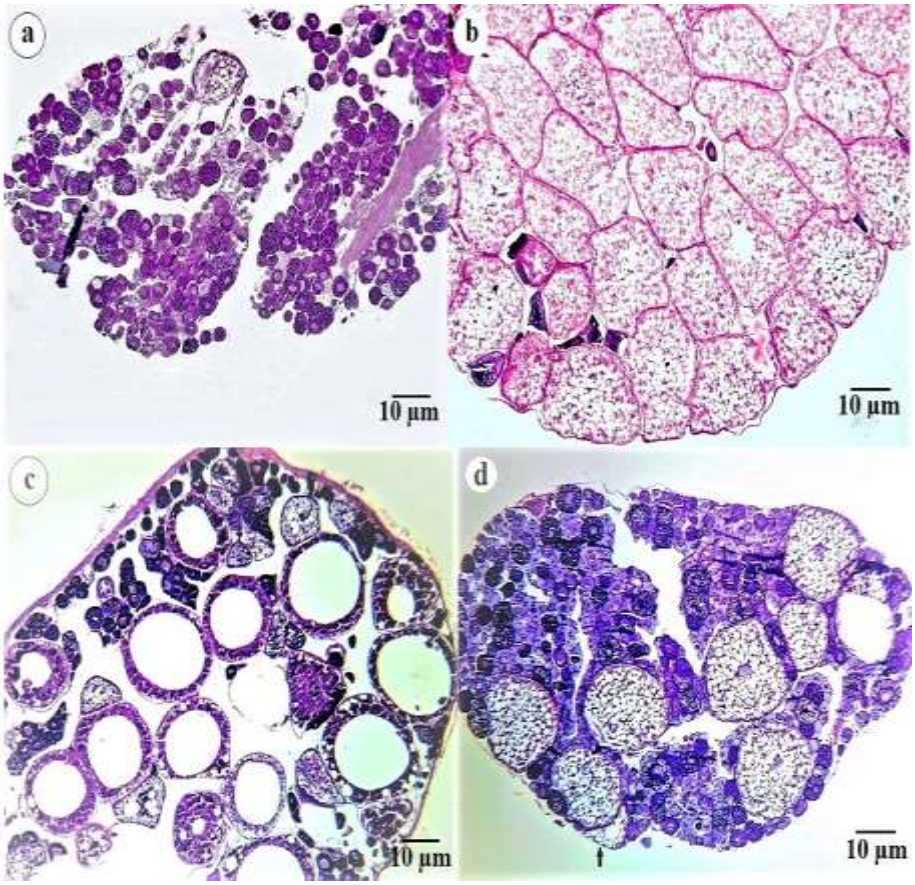
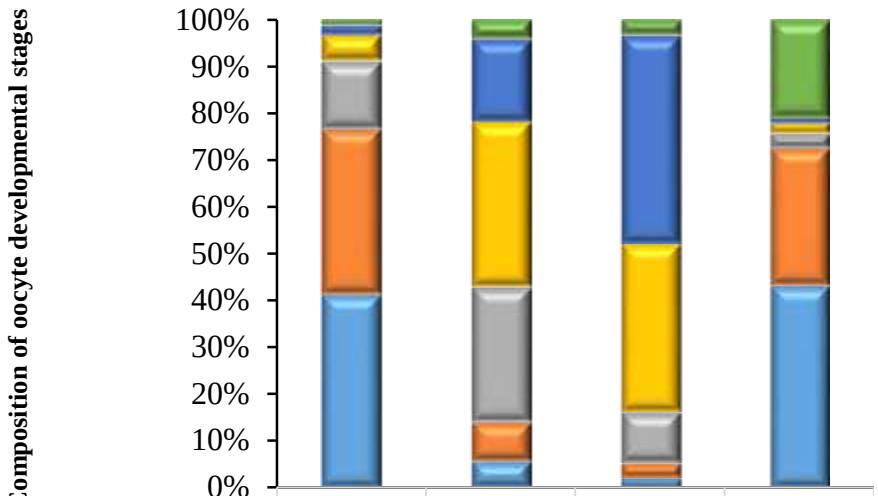


Fig. 11 Representative photomicrographs of ovaries in *Anabas testudineus* showing different stages of reproduction (a) Preparatory ovary; (b) Prespawning ovary; (c) Spawning ovary; (d) Postspawning ovary



	Preparatory (PRE)	Pre-spawning (PRP)	Spawning (SP)	Post-spawning (PSP)
Atretic	1.128668172	3.982300885	3.254972875	20.72072072
Mature	2.031602709	17.69911504	44.48462929	1.261261261
Vitellogenic	5.643340858	35.39823009	35.98553345	2.342342342
Previtellogenic	14.44695526	28.76106195	10.84990958	3.063063063
Perinucleolus	35.44018059	8.407079646	3.254972875	29.36936937
Chromatin nucleolus	41.30925508	5.752212389	2.169981917	43.24324324

Fig. 12 Composition of oocytes during different stages of gonadal development (n = 10 frame/stage)

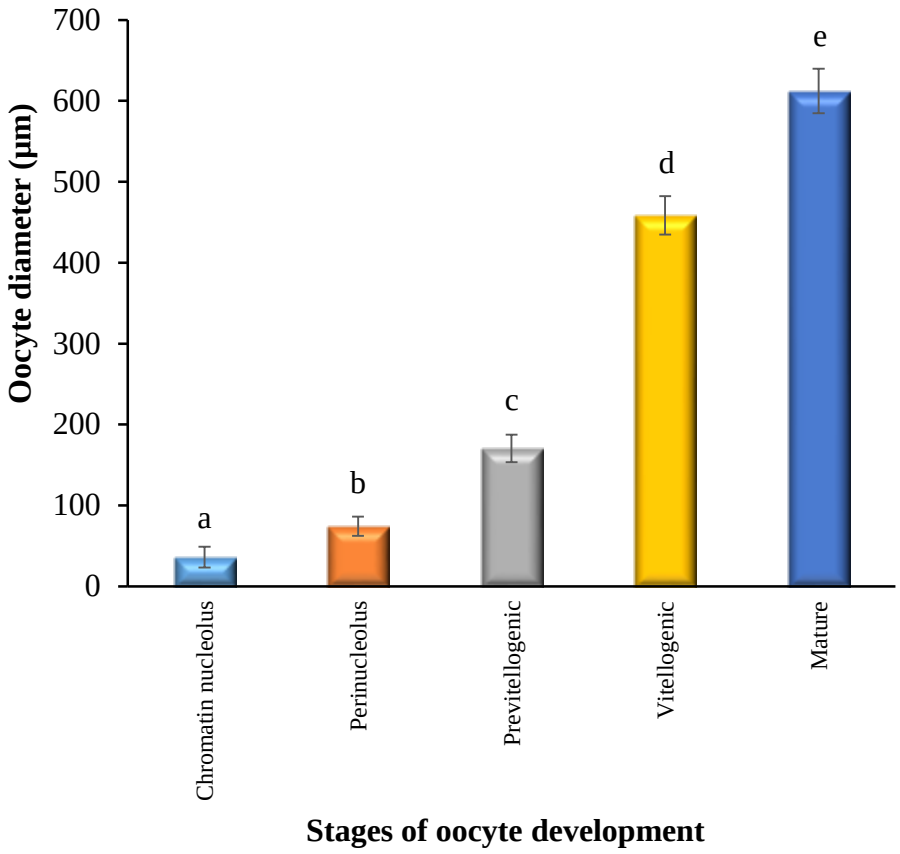


Fig. 13 **Size of oocytes at different stages of gonadal development (Mean ± SD; Significant (P<0.05) differences among means are indicated by different letters in superscript)**

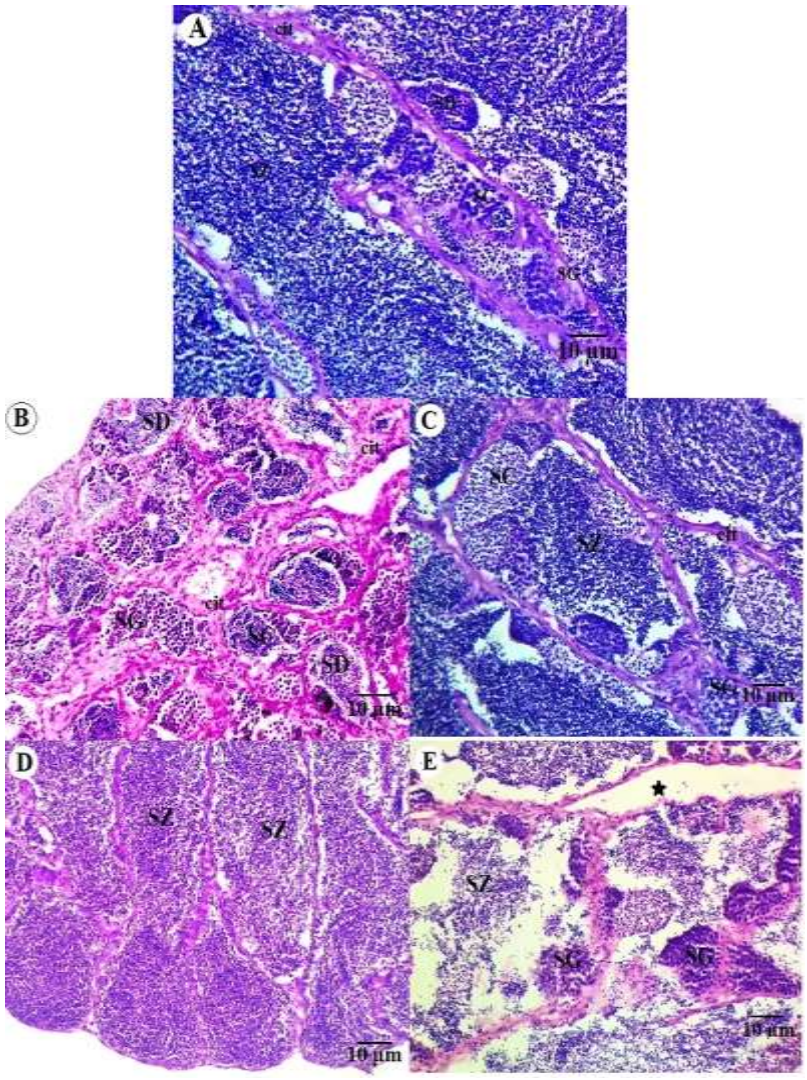
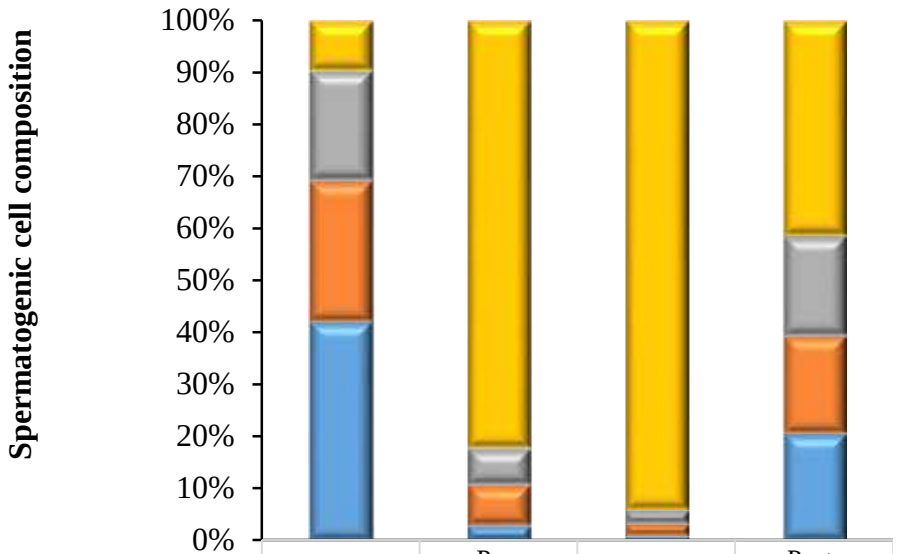


Fig. 14 Representative photomicrographs of testis in *Anabas testudineus* showing different stages of gonadal development (A) Testis of all stages; (B) Preparatory stage; (C) Prespawning stage; (D) Spawning stage; (E) Postspawning stage, Spent testis with loosely packed spermatozoa inside the seminiferous tubule; SG-Spermatogonia; SC-Spermatocytes; SD-Spermatids; SZ-Spermatozoa; cit-Connective Interstitial tissue; Empty seminiferous tubule (asterisks)



	Preparatory (PRE)	Pre-spawing (PRP)	Spawning (SP)	Post-spawning (PSP)
Spermatozoa (SZ)	9.603600317	82.05552871	93.92709298	41.1047705
Spermatids (SD)	21.0344181	7.057234029	2.73775538	19.32663502
Spermatocytes (SC)	27.24662527	7.875123563	2.312012436	18.89103992
Spermatogonia (SG)	42.11535631	3.012113701	1.0231392	20.67755456

Fig. 15 Composition of spermatogenic cells during different stages of gonadal development (n = 10 frames/ stage)

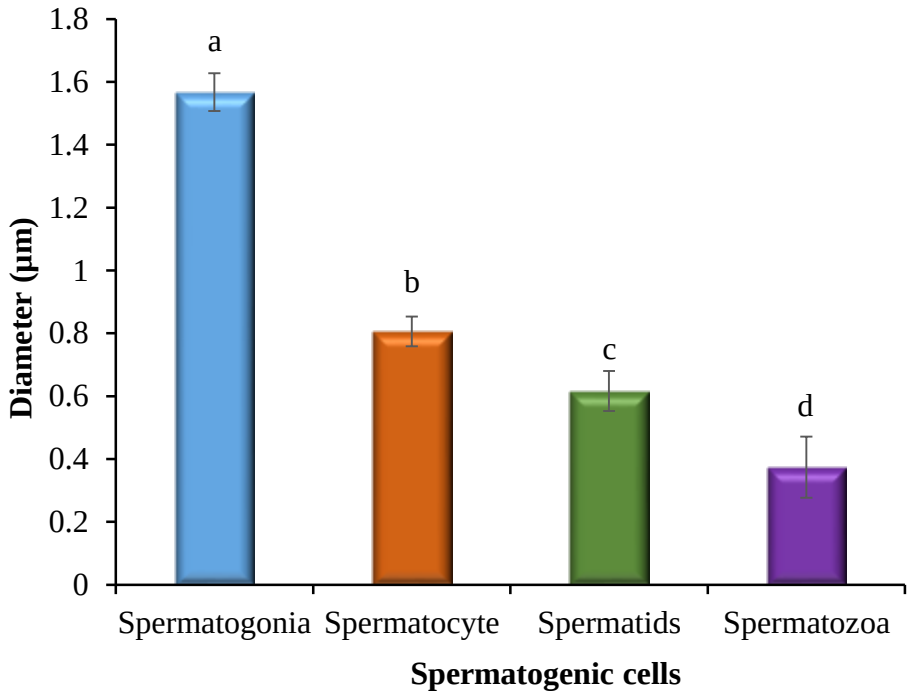


Fig. 16 **Size of testicular cells during different stages of gonadal development (Mean ± SD; Significant (P<0.05) differences among means are indicated by different letters in superscript)**

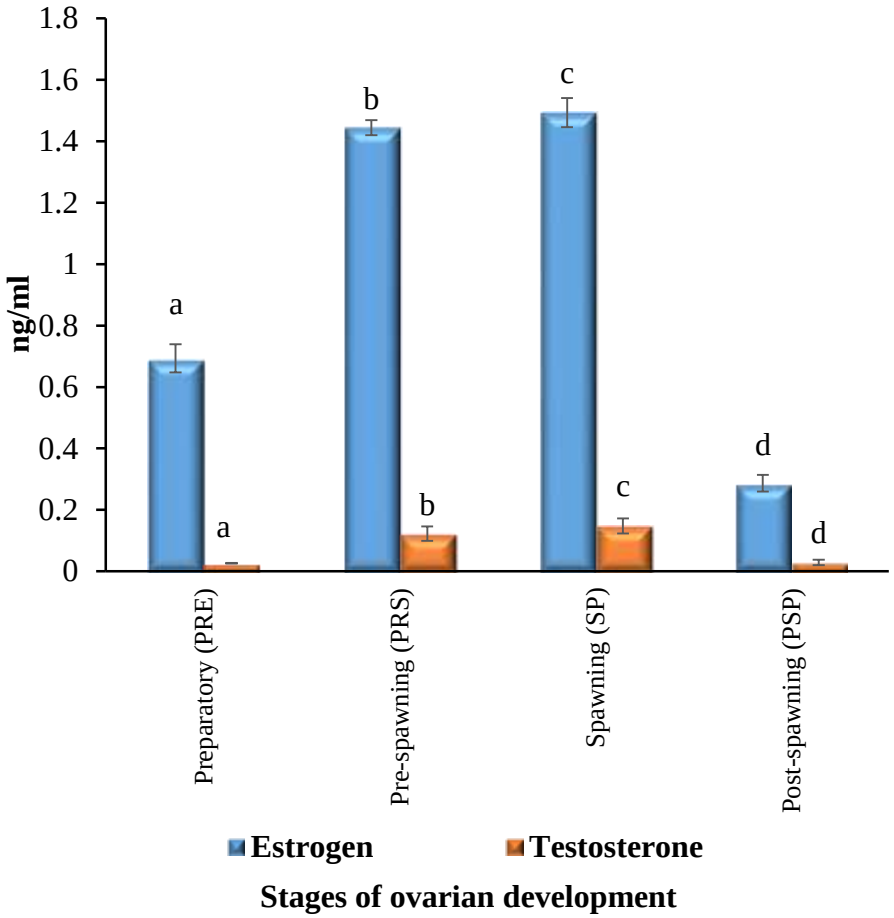


Fig. 17 **Levels of sex hormones during different stages of ovarian development in *Anabas testudineus* (Mean ± SD; n = 10/ stage, in replicates; Significant (P<0.05) differences among means are indicated by different letters in superscript)**

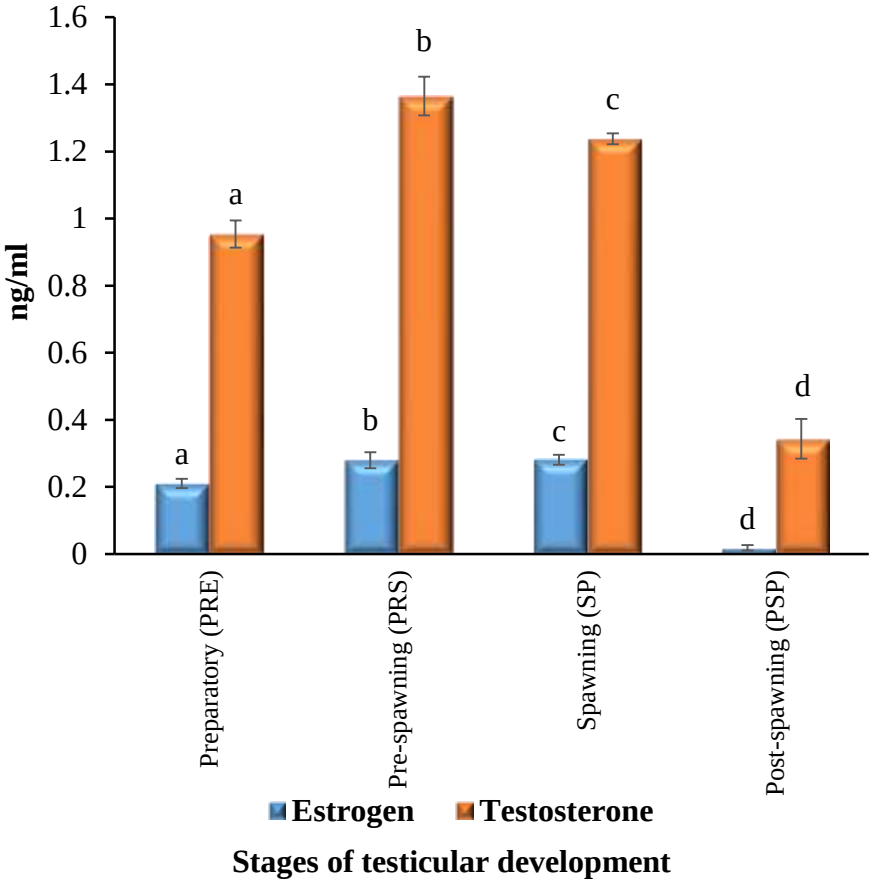


Fig. 18 **Levels of sex hormones during different stages of testicular development in *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant (P<0.05) differences among means are indicated by different letters in superscript)**

6. DISCUSSION

6.1 Phylogenetic analysis of *Anabas testudineus*

Fish phylogenetics is extensively studied using mitochondrial DNA (mtDNA) as it evolves more rapidly than the nuclear DNA, and accumulates the variation between the closely related species (Patwardhan *et al*., 2014). The mitochondrial 16S rRNA gene and the protein-coding cytochrome C oxidase subunit I (COI) genes are highly conserved and used as the most popular marker in molecular systematics (Lakra *et al*., 2008). The 5' portion of the COI gene consisting of approximately 640 nucleotides is widely used by the DNA barcoding consortium for the unique identification and the discovery of many species (Hebert *et al*., 2003; Stoeckle, 2003; Moritz and Cicero, 2004).

The present study was conducted to explore the molecular identification of the genus Anabas collected from two sites, namely Pulimugham Fish farm of Alappuzha, and local water bodies of Kozhikode district, Kerala, India. The amplified partial nucleotide sequences of the COI gene isolated from Alappuzha and Kozhikode sites have 612 and 683 base pairs, respectively, thereby showed genetic variations. In India, two species of Anabas have been identified namely *Anabas testudineus*, Bloch, 1795 and *Anabas oligolepis*, Bleeker, 1855 (Rao, 1968; Srinu *et al*., 2019). The morphological identification of these species has some limitations as they are found to differ only based on their body depth and size. The body of *A.*

oligolepis has been found more compressed, and comparatively smaller in size while *A. testudineus* possesses a less deep body, longer pectorals, short snout, and dark spot at the base of the caudal fin fading with age (Srinu *et al*., 2019). Karyotype analysis revealed that *A. oligolepis* consists of 46 chromosomes whereas *A. testudineus* possess 48 chromosomes (Dutt and Ramaseshaiah, 1980).

The molecular approach using sequences of COI gene proved effective for the identification and phylogenetic analysis of Anabas species collected from two sites of Kerala. The common feature of Anabas species obtained from two sites includes the codon structure of the COI gene, which was CT-biased, however, the nucleotide frequencies showed variations. The number of occurrences of A, T, C, G of COI gene of Anabas obtained from the Alappuzha site were 23.37%, 31.37%, 28.76%, and 16.5%, respectively. Meanwhile, the nucleotide frequencies of the COI gene of sequenced Anabas from the Kozhikode site were 22.99% for A, 30.75% for T, 29.28% for C, and 16.98% for G.

The phylogenetic tree constructed using Maximum Likelihood and Neighbor-Joining method showed two major clades, in which the Anabas species of Alappuzha site were found similar to those species identified from Bangladesh, Vietnam, and Malaysia whereas Anabas of Kozhikode site resembled closer with the species identified from Kochi of India, Philippines, France, and Indonesia. Besides, another subclade of *A. testudineus* reported from West Bengal, India shared a sister-clade relation with the species obtained from the Alappuzha district. The genetic divergence analysis for intra-species found no

divergence among *A. testudineus* from the Alappuzha site while the differences were more pronounced among the species collected from the Kozhikode site. Thus present study selected *A. testudineus* obtained from the Alappuzha district, as they were found close together with the COI gene reported from the other Asian countries.

6.2 Morphological identification of sex and gonadal stages

Morphological identification of sex in fish is a very crucial process as they lack well-defined external genital organs. Like higher vertebrates, some fish species exhibit sexual dimorphism with distinct morphological features. Some of the secondary characters used during spawning such as claspers, papillae, the gonopodium, etc., serve to identify the sex externally, without sacrificing the fish. Aquaculture practices depend on the secondary sexual characters for the artificial breeding procedures and are subsequently associated with gonadal or sex differentiation. *Anabas testudineus* do not exhibit sexual dimorphism during the non-breeding season, while the identification of sexes mostly relies on the internal examination of gonads, but remains challenging in immature fish.

The body color, belly structure, and position of the vent contribute to distinguishing the sex during the breeding season (Lagler *et al.*, 1977; Behera *et al.*, 2015b). The present study observed a remarkable change in the body color during the breeding season i.e., from April to August, with an appearance of the reddish-yellow color on the ventral surface of the mature fish. The belly of both sexes remained the same during the non-breeding season due to less volume

of gonads. However, during the onset of the breeding season, a gradual increase in belly size was observed, indicating the development of gonads. In the breeding season, female fish were identified by a bulged belly since it contained bilobed, vitellogenic ovary with mature ripen oocytes.

The vent opening found beneath the anal fin on the ventral side of the fish often referred to as the genital papilla or ovipositor in females is used in sex differentiation. During the breeding season, the vent of female fish exhibited a red colored distinct outgrowth, which was more prominent upon a slight pressure on the abdomen but such structure remained absent in male fish. There was no remarkable difference in the vent color and no outgrowth was observed in both sexes during the non-breeding season. Similar sexual dimorphic changes have been reported in both sexes of the fish during the breeding season in the natural environment (Behera *et al*., 2015b). Since *A. testudineus* did not exhibit sexual dimorphism during the non-breeding season, the abdomen was cut open to identify different developmental stages of gonads. The present study conducted in the laboratory condition identified different gonadal stages such as preparatory stage from February to March, pre-spawning stage from April to June, spawning stage from July to August, and post-spawning stage from September to January, which was further confirmed by histomorphological analysis, and discussed in detail in the later sections.

6.3 Length and weight of the fish

The growth performance of *A. testudineus* was evaluated in terms of length and weight of the fish during different stages of gonadal development, and categorized as preparatory, pre-spawning, spawning, and post-spawning stages. Both male and female fish showed a progressive increase in the average weight and length from the preparatory to post-spawning period. The present study observed that fish in the non-breeding season were found to be more active, and they invest the excess energy for swimming activity. However, fish during the breeding season remained sluggish, where it was known to utilize their energy for spawning and other reproductive activities. The study expected a drastic increase in average weight during the spawning period but the results showed only a progressive increase right from the preparatory to post-spawning period.

Generally, fish species suppress their growth performance by reallocation of energy in the form of catalyzing endogenous nutrients to meet energy demand for better reproductive performance (Coward *et al*., 2002). Similar to the current study, a trend of energy reallocation from body growth to the maintenance of normal reproductive functions has been reported in *Astyanax aff. bimaculatus* in the natural environment (Araujo *et al*., 2019). Some studies have reported that in the natural environment, *A. testudineus* of size from 8 to 9 cm attained the first sexual maturity in both sexes (Jacob, 2005; Khatun *et al*., 2019). However, another study has observed that in the controlled laboratory condition, males of approximately 6 cm length and 4 g weight, and females of about 7 cm length and 7 g weight attained

gonadal maturity (Ndobe *et al.*, 2020). The present study observed that *A. testudineus* of nearly around 7 cm length and 7 g weight attained gonadal maturity in the laboratory condition. The evaluation of growth performance in response to gonadal maturity serves widely in aquaculture practices for the maintenance and management of fish stock in the natural environment.

6.4 Absolute and relative weights of liver tissues

One of the highly sensitive and accurate ways to assess the health status of fish is to determine the absolute and relative weights of liver tissues. Similar to the higher vertebrates, the fish liver is the major tissue involved in metabolism, secretion of digestive enzymes, and the biotransformation or detoxification processes. Besides, the fish liver plays an important role in vitellogenesis where it synthesis a precursor of the egg yolk protein namely the phosphoglycoprotein vitellogenin, and release through the bloodstream to reach the developing oocytes (Moussavi *et al*., 2009). Thus the study of absolute weight of liver tissue as well as the relative weight or hepato-somatic index (HSI) was performed to assess the role of liver tissue in fish reproduction.

The present study measured the absolute and relative weights of liver tissue every month throughout the year, and also recorded during the different stages of reproduction. Both in the month-wise and reproductive stage-wise estimation of liver weights in both sexes showed a remarkable increase from December to March, which was the late post-spawning to the preparatory stage. The HSI also showed a

similar increase from December to March in female and male fish, which were identified as post-spawning and preparatory stages of reproduction. HSI has been associated with the energy reserves and metabolic activity of liver tissues and is often used as a biomarker to indicate fish metabolism. The increase in liver weights observed during the non-breeding season could be due to the continuous biosynthesis and trafficking of vitellogenin protein for the development of oocytes, which is essential for spawning and other reproductive functions (Araujo *et al*., 2019). This was further confirmed by the decline in the absolute and relative weights of liver tissues during the breeding season, i.e., in the pre-spawning and spawning periods as the energy reserves could be utilized by the fish for gonadal maturation (Larson and Gupta, 2019). Similar fluctuations in HSI with the highest peak during the post-spawning and preparatory period along with the minimum range during pre-spawning and spawning phases have been reported in the catfish *Heteropneustes fossilis* and *Melanotaenia boesemani* (Singh and Srivastava, 2015; Hismayasari *et al*., 2015).

6.5 Absolute and relative weights of gonads

The reproductive activity of *A. testudineus* was assessed by measuring the absolute and relative weights of gonads, the ovary, and the testis. The GSI was generally considered as indices of reproduction since they provide an indirect means of various ecological effects in the natural environment. Hence used as a crucial factor to determine the male and female reproductive conditions in fish (El-Sayed and

Kawanna, 2007). The present study observed a notable increase in the absolute and relative weights of gonads or gonadosomatic index (GSI) of the fish from April to August, i.e., in pre-spawning and spawning stages of reproduction.

The maximum GSI attained during the breeding season of female fish evidenced the presence of mature vitellogenic ripen ovary, which was actively involved in spawning whereas the decrease in GSI values observed during the non-breeding season indicated the post-spawning and preparatory stages. GSI is widely used as an indicator to determine the progression of gametogenesis in fish, particularly used to identify the period and timing of spawning. The developing oocytes with the rise in GSI during the gonad maturation could be due to the deposition of large amounts of lipids and proteins (Patino and Sullivan, 2002). In the present study, an unsuccessful spawning was noticed under laboratory conditions, and this could be due to the hormonal fluctuations that initiate the spawning process (Mylonas and Zohar, 2001; Zohar and Mylonas, 2001).

The present study showed variations in the levels of GSI in male fish during different stages of reproduction. Besides, the female fish exhibited a high percentage of GSI than the males, which was documented by the size of gonads. Similar results have been reported in *Cyprinus carpio* and *Ctenopharyngodon idella* under the composite culture system with a special reference to pond fertilization (Mahboob and Sheri, 2002). Moreover, an increase in GSI observed during the pre-spawning and spawning stages indicated that *A. testudineus* is a seasonal breeder (Bernal *et al*., 2015).

The deposition of vitellogenin yolk protein occurred during the pre-spawning and spawning periods of the reproductive cycle, which could be the reason for an increase in GSI. Similar to the present study, the physiology of developing gonads showing an increase in GSI during the breeding season has been found to be inversely correlated with the percentage of HSI in some fish species such as *Catla catla*, *Labeo rohita*, *Cirrhina mrigala*, *Hypophthlamichthys molitrix*, *Ctenopharyngodon idella*, and *Cyprinus carpio* (Mahboob and Sheri 2002). Thus the modulations in the percentage of HSI along with GSI have been considered as useful indicators to evaluate the reproductive fitness of the species (Pandit and Gupta, 2019).

6.6 Fecundity in *Anabas testudineus*

Fecundity or egg-laying capacity is the physiological process used to measure the reproductive potential of the fish by determining the number of ripening eggs produced during the spawning season (Bromage *et al*., 1992). Fecundity is an important factor in fish stock management where it quantifies the reproductive capacity of individual fish, but sometimes shows variations even within the stock. The present study estimated that the fecundity rate of *A. testudineus* varied between 6,500 and 11,000 eggs per unit of ovary weight using the conventional gravimetric method. The relationships between the fecundity and body weight, ovary weight, ovary length, ovary volume, and fish length have been widely analyzed to understand the features of the reproductive biology in fishery management (Ziauddin *et al*., 2016).

The present study observed a high degree of positive correlation, i.e., r = +0.9528 between the number of eggs produced and body weight of the fish, ranging from 7.5 to 9.5 g with the regression equation of y = 1050.5x; R^2 = 0.8928. In another study, the fecundity of *A. testudineus*, weighing 30-67 g and 12 to 16 cm size, varied between 12,355 and 41,820 eggs (Banu *et al*., 1985). Similarly, the female climbing perch of the mean body mass ranging from 44 to 78 g showed the mean fecundity as 24,120 eggs (Amornsakun *et al*., 2005) while the fish of size 32-50 g had produced about 5,979 to 13,565 eggs (Ziauddin *et al*., 2016). In the present study, the relationship between fecundity and ovary weight also showed a high degree of positive correlation. The ovary weight that exists between 900 and 1800 mg during the spawning period showed the correlation co-efficient as r = +0.9353 with the regression equation of y = 6.2803x and R^2 = 0.6557. The findings were in agreement with another study showing a similar positive degree of correlation with r value as +0.8642 for *A. testudineus* possessing an ovary weight between 80.5 and 586 mg (Ziauddin *et al*., 2016).

Anabas testudineus of the length between 6.5 and 9 cm showed a positive correlation, i.e., r = +0.9024 with the regression equation of y = 1093x, and the coefficient of determination, R^2 = 0.8848. Another study on *A. testudineus* has also reported that the relationship between fecundity and body weight, body length, and ovary weight showed a linear and positive correlation, and suggested that some environmental factors such as location, water quality, nutritional status, and food

availability could influence the rate of fish fecundity (Lawson, 2011; Marimuthu *et al*., 2009).

6.7 Histomorphology of ovary

The ovary of *A. testudineus* is a slightly unequal cylindrical, paired, and lobed structure attached to the dorsal coelomic wall by mesovarium, and opens through a common oviduct leading to the vent (Jacob, 2005). The ovarian wall consists of an outer thin, transparent peritoneal layer called serosa, and inner thick, elastic tunica albuginea containing connective tissues, smooth muscles, and blood vessels. Ovigerous folds are the finger-like foldings that projects from the ovarian wall into the lumen of the ovary or ovocoel, which holds developing oocytes of different stages (Selman and Wallace, 1989; Nishimura *et al*., 2018). According to the shape, size, color, texture, and microscopic observations of the developing oocytes, the developmental stages of fish have been classified as immature, maturing, mature, ripe and spent, represented as stage I to V, respectively (Newton and Kilambi, 1969). However, based on the oocyte development and microscopic observations, the present study identified four distinct developmental stages of oocytes in *A. testudineus* namely preparatory, pre-spawning, spawning, and post-spawning. The observations were in agreement with another study, which reported similar stages of gonadal development in *A. testudineus* (Pal *et al*., 2018). However, in the present study the months representing those stages showed a slight variation.

The microscopic analysis identified that the first immature stage of *A. testudineus* occurred from February to March as the preparatory stage, consisting of both chromatin nucleolus and perinucleolus oocytes. The chromatin nucleolus contained large centrally located nucleoli while the perinucleolar oocytes were identified by the presence of several nucleoli at the periphery of the nucleoplasm (Wang *et al*., 2017). The histological analysis performed during April to June recognized the pre-spawning stage possessing pre-vitellogenic and vitellogenic oocytes. The cortical alveolus began to develop on the periphery of the ooplasm with abundant yolk granules to become pre-vitellogenic oocytes, while the vitellogenic oocytes contained numerous lipid droplets. A similar observation has been reported in the zebrafish, *Danio rerio* during its reproductive development (Koc *et al*., 2008).

The spawning stage was identified during July and August as evidenced by the presence of mature oocytes with loosely arranged, and centrally located large oil vacuoles showing a remarkable increase in the size of oocytes. The last stage of oocyte development in *A. testudineus* was the post-spawning stage, which occurred from September to January consisting of both atretic and spent oocytes along with a very few developing immature oocytes. Atresia is a normal physiological event characterized by the disintegration of the nucleus, breakdown of the vitelline envelope, and degeneration of yolk granules and follicular cells, influenced by several factors such as alterations in the levels of hormones, environmental conditions,

temperature, photoperiod, nutritional supply, and water quality (Miranda *et al*., 1999).

In the breeding season, the spawning ovary of *A. testudineus* contained abundant mature oocytes with some developing immature oocytes thereby indicated a group-synchronous type of ovarian development. The morphometric measurement revealed that the diameter of the mature ova in *A. testudineus* ranged between 599-625 µm, while another study reported that the ova diameter of *A. testudineus* collected from the water bodies of Malaysia varied between 0.54 and 0.80 mm of the fish weighing 36.84 to 105.26 g and 12.4-19.2 cm length (Marimuthu *et al*., 2009).

Similarly, another study has observed a group-synchronous oocyte development in some neotropical freshwater fishes supported by the short spawning season between July and August, and a quick reduction of GSI immediately after spawning (Rizzo and Bazzoli, 2020). In group synchronous development, two cohorts of developing oocytes have been observed that include a fairly synchronous population of larger oocytes, and a heterogeneous population of smaller immature oocytes. The large mature oocytes have been found to be spawned in the nearest breeding period whereas the immature oocytes are stored for future spawning (Murua *et al*., 2003).

6.8 Histomorphology of testis

The testis of *A. testudineus* is a paired, elongated structure attached to the dorsal coelomic cavity by a thin membranous layer known as mesorchium that joins caudally and converges into

spermatoduct, which finally opens to the exterior through the urogenital pore (Uribe *et al*., 2015). Tunica albuginea covers the testis, which then protrudes into the testicular parenchyma as numerous seminiferous lobules comprising different stages of testicular cells (Schulz *et al*., 2010). In the present study, histological analysis revealed the lobular type of testis in *A. testudineus* formed of many cysts-like grouped seminiferous tubules as identified in the Denison barb, *Sahyadria denisonii* from the rivers and streams of the Western Ghats of India (Sajan and Mercy, 2016). During development, the growth of testis was accompanied by the gamete formation and production of viable sperm. Spermatogenesis in fish is a highly organized and coordinated process that characterizes morphologically distinct germ cell stages such as spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa (Schulz *et al*., 2010).

Based on the species and spawning season, several stages of testicular development have been identified in different freshwater fishes, which determine the testicular maturity (Rutaisire *et al*., 2003; Montchowui *et al*., 2012). Similar to females, the annual reproductive cycle of males observed in the present study includes four stages such as preparatory or resting, pre-spawning, spawning, and post-spawning (Golpour *et al*., 2021). The preparatory stage identified during February to March contained a thin, delicate, and transparent testis having primarily of spermatogonia. The pre-spawning and spawning stages that occurred during April to June, and July to August showed a gradual increase in the composition of spermatozoa. Morphological

identification revealed a well-distinguished thick, flattened, and creamy white colored testis. The post-spawning testis was characterized by loosely packed spermatozoa and empty lumen in the constricted seminiferous tubules. A similar pattern of testicular development has been reported in *A. testudineus* collected from the water bodies of the Philippines (Bernal *et al*., 2015), and in *Sahyadria denisonii* from Western Ghats of India (Sajan and Mercy, 2016).

Spermatozoa were observed throughout the year but were found in an abundance of 93.9% during the spawning season, with an average size of 0.37 µm in diameter suggesting a group-synchronous development in *A. testudineus*. Thus the present study demonstrated that spermatogenesis occurred seasonally in *A. testudineus*, and showed a peak production during the breeding season.

6.9 Gonadal hormones

The hypothalamo-pituitary-gonadal (HPG) axis regulates the reproduction of teleosts by the release of gonadotropin-releasing factor from the hypothalamus in a pulsatile fashion, which stimulate pituitary for the secretion of gonadotropins namely luteinizing hormone and follicle-stimulating hormone. Gonadotropins from the pituitary act on the gonads for the release of sex steroids such as estradiol in females and testosterone in males (Meethal and Atwood, 2005). Sex steroids were involved in the development, maintenance, and functioning of the reproductive processes. In the present study, the level of estradiol in the blood serum of female fish increased during pre-spawning and spawning stages of ovarian development along with the slight increase

in the level of testosterone. The rise in the level of estradiol during the spawning period has been shown to be responsible to trigger the liver for the production of vitellogenin, which was later incorporated into the developing oocytes (Pelissero *et al*., 1993).

The present results also revealed an increase in the level of testosterone in male fish during the pre-spawning and spawning period of testicular development, along with the reduction in the level of estradiol stating the role of testosterone in spermatogenesis during the breeding season. The level of testosterone also coincided with the increased GSI during the breeding period as reported in the male golden rabbitfish *Siganus guttatus* under captivity (Pham and Le, 2020). Another study also reported a rise in the levels of testosterone during the pre-spawning period in the male catfish *Heteropneustes fossilis* (Chaube *et al*., 2018). Thus the evaluation of gonadal hormones provided a piece of key information about certain reproductive parameters such as gonadal differentiation, gametogenesis, steroidogenesis, and fecundity to predict the reproductive success of the fish, *A. testudineus*.

7. CONCLUSIONS

1. The phylogenetic analysis of *Anabas testudineus* using mitochondrial cytochrome oxidase I (COI) gene distinguished the species collected from Alappuzha and Kozhikode districts, Kerala, India, and selected the species of Alappuzha for further studies as they resemble close to those species found in other Asian countries.
2. *Anabas testudineus* did not exhibit sexual dimorphism, however during the breeding season bulged belly, and red-colored vent with an outgrowth were prominent in the females.
3. The fish is a seasonal breeder with a group synchronous type of gonadal development in both sexes.
4. Based on gonadosomatic index, composition of germ cells, fecundity, level of sex steroids, and gonadal histology, four different stages of gonadal development was identified in the laboratory condition such as preparatory stage from February to March, pre-spawning stage from April to June, spawning stage from July to August, and post-spawning stage from September to January.
5. Both male and female fish attained gonadal maturity nearly around 7 cm length and 7 g weight, and showed a progressive growth performance from the preparatory to post-spawning period.

6. The absolute and relative liver weights or hepatosomatic index in both sexes showed a remarkable increase from September to March, i.e., in the late post-spawning to the preparatory stage, and this could be due to the continuous biosynthesis and storage of energy reserves.
7. The absolute and relative weights of gonads or gonadosomatic index increased from April to August, i.e., in pre-spawning and spawning stages of reproduction, which indicated the deposition of large amounts of lipids and proteins in the developing oocytes.
8. The fecundity rate of *A. testudineus* varied between 6,500 and 11,000 eggs.
9. The histomorphology of ovary and testis distinguished different developmental stages.
10. The rise in the levels of estradiol in females and testosterone in males during the breeding season accounts for the gonadal differentiation, gametogenesis, steroidogenesis, and fecundity of the fish, *A. testudineus*.

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CHAPTER 3

Acute toxicity of triclosan in the fish, *Anabas testudineus*

Chapter 3 **Acute toxicity of triclosan in the fish, *Anabas testudineus***

CONTENTS

LIST OF ABBREVIATIONS

%	Percentage
A	Adenine
ADI	Acceptable daily intake
ASTMI	American Society for Testing and Materials International
bp	Base pair
BSI	British Standards Institute
C	Cytosine
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
COI	Mitochondrial cytochrome oxidase I
CSA	Canadian Standards Association
d	Days
DMSO	Dimethyl sulfoxide
DNEL	Derived no-effect level
DT_{50}	Half-life of a compound
EC_{50}	Median Effective Concentration
ECHA	European Chemicals Agency
ERA	Environmental risk assessment
G	Guanine
g	Gram
h	Hour
ISO	International Organization for Standardization
Kow	Octanol-water partitioning coefficient
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LOAEL	Lowest Observed Adverse Effect Level
mbp	Myelin basic protein
MEGA	Molecular Evolutionary Genetics Analysis
mg L^{-1}	Milligram per litre
µg L^{-1}	Microgram per litre
mg/kg bw/d	Milligram per kilogram bodyweight per day
MS	Microsoft
mtDNA	Mitochondrial Deoxyribonucleic acid
n	Number of samples

NCBI	National Centre for Biotechnological Information
No.	Number
NOAEC	No Observed Adverse Effect Concentration
NOAEL	No Observed Adverse Effect Level
NOEC	No Observed Effect Concentration
OECD	Organisation for Economic Co-operation and Development
OEL	Occupational exposure limit
P	Probability value
PEC	Predicted environmental concentration
pH	Potential of hydrogen
PNEC	Predicted no-effect concentration
ppm	Parts per million
RfD	Reference dose
RQ	Risk quotient
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
syn2a	Synapsin IIa
T	Thymine
US EPA	United States Environmental Protection Agency
US FDA	United States Food and Drug Administration
WHO	World Health Organization

LIST OF TABLES AND FIGURES

1. INTRODUCTION

1.1 Overview on aquatic toxicity

Aquatic toxicology predicts the effects of contaminants in ecosystems and organisms through different toxicity studies. Aquatic toxicology is defined as "*the study of the effects of natural and anthropogenic materials on the subcellular to individual aquatic organisms to communities and ecosystems*" (Rand, 1995). Aquatic toxicology is a multidisciplinary field that integrates toxicology, aquatic chemistry, and ecology including marine, freshwater, and other water bodies. The route of entry of the toxicants into various organisms like microbes, invertebrates, and vertebrates, and their subsequent adverse effects at the individual level to ecosystem level is predicted using several testing batteries (Jones and Lassiter, 2020). There are different tools and methods to monitor the adverse effects of the toxicants, and the data generated are widely used for their risk assessment in the aquatic environment, and the well-being of humans.

Ecotoxicological studies have been practiced for using two basic assessment methods such as retrospective and predictive approaches. In the retrospective approach, the toxicity assessment is carried out using the existing observations to generate scenarios about the future, thereby sources of contamination can be identified to prevent similar discharges into the environment. However, in the predictive approach, the response of the contaminants in the environment is predicted using different models in the laboratory

conditions before they appear in the natural environment, and it is widely used as a supplementary source for decision making, and environmental policies (Nikinmaa, 2014).

1.2 Environmental risk assessment

The term environmental risk assessment (ERA) covers the assessment of the impacts of various environmental stressors like diseases, xenobiotics, invasive species, climate change, etc., on the structure, functions, and biodiversity of the ecosystem (Franco *et al*., 2006). The ERA is associated with risk management by identifying the threat to the environment or human health and imposing proper measures to control and manage the risk. Risk management consists of two basic concepts such as the 'effects-oriented approach' and 'source-oriented approach' to determine the magnitude of the potential adverse effects of the toxicants (Chaumot *et al*., 2014).

The key steps involved to evaluate the risk assessment processes include problem formulation, hazard identification, hazard characterization, exposure assessment, and risk estimation (Calow, 1998). During the first step, the effects of toxicants on the ecosystem and organisms are recognized based on laboratory experiments or field data. The consequences of toxicants, their interactions with the biotic and abiotic factors in the environment, and susceptibility at the species level are identified in the hazard identification. The detailed information on the adverse effects and mode of action of the toxicants on different species derived by several toxicological testing demonstrates the hazard characterization. Exposure assessment

estimates the probability of the consequences, while the severity of the consequences is evaluated using risk estimation (Nikinmaa, 2014).

Finally, the risk assessment is performed by calculating the value of the risk quotient (RQ), which is the ratio of predicted environmental concentration (PEC), and the highest predicted no-effect concentration (PNEC) of a toxicant in the environment. PEC is the potential risk of the toxicant to the environment whereas PNEC is the concentration of the chemical below which no adverse effects are expected to occur in the environment, which is based on the median lethal values of the toxicant. Thus the acute toxic risk of a chemical in the aquatic environment can be predicted through ERA using the RQ value. It has been proposed that if the value of RQ lies below 0.1 is at insignificant risk, 0.1-1 represents low risk, while 1-10 has moderate risk, and above 10 denotes high risk (Ollala *et al*., 2018; Chavoshani *et al*., 2020).

1.3 Toxicity testing

Toxicity testing can be performed by following specific methodologies including prioritization of risks, site-specific risk evaluation, comparative risk assessment, and quantification of risks. The risks of the toxicant in the environment are prioritized by evaluating their adverse effects on the biological endpoints such as survival, growth, behavior, reproduction, and other physiological aspects of the test organisms in the laboratory conditions (Telego and Rothenberg, 1996). The assessment technique identifies the potential chemicals that harm the ecosystems, and are categorized under the

prioritized toxicant. The site-specific risk evaluation helps to determine the risk of the toxicant associated with a specific location, and it is also referred to as 'environmental site assessment' (Anderson *et al*., 2004).

In the comparative risk assessment, the comparison of the relative risks of more than one toxicant are evaluated, and often compared with the unpolluted biota or organisms. All kinds of toxicity testing are quantified to identify the risks of toxicants to establish and implement proper measures for the control or management of pollutants in the environment. Most of the environmental risks of the aquatic toxicants are established primarily through the concentration-effect relationship. The effects are mainly based on several factors such as solubility of the compound, physico-chemical properties of the toxicant, mode of exposure, dosage and duration of exposure, target organisms, etc. Hence toxicity testing has numerous benefits that significantly contribute to the ecological risk assessments processes in multiple ways, including identification of the occurrence of toxicants in the environment, distribution of the toxicant, combined effects of multiple compounds, effects of toxicity such as lethal or sublethal thereby helps for the decision-making processes (Eaton *et al*., 2018).

The effectiveness of toxicity testing is further monitored under the definite standard principles, rules, guidelines, and practices developed by various governmental, non-governmental, or quasi-governmental organizations. It includes the International Organization for Standardization (ISO), American Society for Testing and Materials International (ASTMI), Clinical and Laboratory Standards Institute (CLSI), British Standards Institute (BSI), and Canadian

Standards Association (CSA). Besides, some of the global, regional, or country-specific organizations that establish guidelines for toxicity testing include the Organization for Economic Co-operation and Development (OECD), the World Health Organization (WHO), the European Chemicals Agency (ECHA), the United States Environmental Protection Agency (US EPA), and the United States Food and Drug Administration (US FDA), etc.

1.4 Methods for validation of toxicity

Several guidelines, standards, and principles proposed by different organizations suggest a broad spectrum of tests that differs with the selection of model organisms, medium and mode of exposure, and different protocols of testing, which includes scientific, product-oriented, or regulatory. Among these, regulatory testing has its own merits in the environmental risk assessment as it plays a significant role in decision-making processes, risk assessment on the organisms, and establishment of water quality standards (Wilson *et al*., 2001). The common and the most effective endpoints to measure the toxicity of a compound are lethal and sublethal effects, which are widely assessed by short-term and long-term toxicity tests that include acute, sub-acute, sub-chronic, and chronic tests (Chinedu *et al*., 2015).

1.4.1 Acute toxicity tests

It is the measure used to test the adverse effects of the toxicant within a short duration after a single dose of exposure. Three routes of exposure namely oral, dermal, and inhalation are used to evaluate the

potential hazards of the chemicals. The effects of the toxicant are usually evaluated by the concentration-mortality response of the organisms. The acute toxicity tests are broadly classified as the median lethal dose or LD_{50} and the median lethal concentration or LC_{50}, at which the toxicant kills 50% of the test animal within the specified duration, i.e., usually 24 to 96 h (Johnson and Finley, 1980). The unit of LD_{50} is expressed as mg of substance per kg of body weight administered per day (mg kg^{-1} bw^{-1} d^{-1}), whereas the unit of LC_{50} is expressed as mg per liter or often in parts per million (ppm).

Besides, there are certain other dose descriptors practiced in the acute toxicity tests to identify the relationship between the specific effects of a chemical substance and the response in the test organisms. It includes No Observed Adverse Effect Level (NOAEL), No Observed Adverse Effect Concentration (NOAEC), No Observed Effect Concentration (NOEC), Lowest Observed Adverse Effect Level (LOAEL), Median Effective Concentration (EC_{50}), and half-life of a compound (DT_{50}), etc. The NOAEL is an exposure level at which no significant increase in the severity of adverse effects between the exposed test population and its appropriate control occurs. Some of the detectable adverse effects include changes in growth, development, morphology, functional capacity, or lifespan of the exposed test organisms (Brody, 2018).

NOAEL is typically obtained after repeated dose toxicity studies or reproductive toxicity studies to derive threshold safety exposure dose to humans such as derived no-effect level (DNEL), occupational exposure limit (OEL), an acceptable daily intake (ADI).

NOAEL values are calculated in a variety of animals for any sexes at a single or combination of chemicals, and also during *in utero* conditions to derive both maternal and embryonic toxicity. The higher NOAEL or NOAEC represents lower systemic toxicity or lower chronic toxicity, and the units are expressed as mg kg^{-1} bw^{-1} d^{-1} or ppm (Rezvanfar, 2014).

NOEC is the highest tested concentration in an environmental compartment for which no significant effects are observed than the control groups. It is obtained from chronic aquatic toxicity studies and terrestrial toxicity studies to identify the chronic environmental hazard classification and to calculate predicted non-effect concentration (PNEC), and the units are expressed in mg L^{-1} (Rajapakse *et al*., 2002). LOAEL is the lowest exposure level at which the adverse effects occur in the exposed organisms when compared to the appropriate control group. It is obtained from repeated dose toxicity studies and reproductive toxicity studies. The EC_{50} value is the concentration of test substances that produce certain effects on 50% of test organisms. They are obtained from aquatic toxicity studies to analyze acute environmental hazard classification and calculation of PNEC and are expressed in mg L^{-1}. Half-life or DT_{50} is the time required by the compound to decline its concentration to half of the initial value through degradation in any environmental compartments such as air, soil, and water. Half-life is used to measure the persistence of a substance in an environmental compartment over a long period (Matthies *et al*., 2008).

1.4.2 Sub-acute toxicity tests

Sub-acute toxicity or repeat dose toxicity is the test to evaluate the adverse effects of the chemical after a repeated administration for a period ranging from 14 to 28 days. The subacute toxicity test is usually conducted to strengthen the toxicity data and to develop the toxicokinetic profile as the study is extended. The study evaluates alterations in clinical biochemistry, histopathology, body weight, food consumption, and hematology. The tests also further extended to evaluate any reversibility of the adverse effects, where all these endpoints are measured after sacrificing the test animal (Denny and Stewart, 2017).

1.4.3 Sub-chronic toxicity tests

A sub-chronic toxicity test is used to establish the effects of the toxicant with repeated exposure for 90 days or within 10% of the animal's lifespan (De Jong *et al*., 2012). They are typically assessed to evaluate the toxic effects at organ, tissue, and cellular level, toxicokinetics of the chemical, study of biotransformation products or the metabolites at various stages of different animal models (Hulla *et al*., 2014). The main purpose of the test is to identify the specific target organs affected by the toxic substances, to establish NOAEL, LOAEL, and effective concentrations, and also to extend the study for chronic toxicity tests. The critical element for the sub-chronic study is to establish a relationship between the adverse effects of the toxicant and the level of exposure on the test animals. The dosage selection for the study is usually performed by the range-finding experiments. The

changes observed in the test animals during the sub-chronic study helps to determine mutagenic, teratogenic, or carcinogenic effects of the test chemicals. The test is frequently used for regulatory purposes by various organizations for fixing the reference dose (RfD) or acceptable daily intake of the chemical (Hulla *et al*., 2014; Eaton and Gallagher, 2010).

1.4.4 Chronic toxicity tests

A chronic toxicity test is used to assess the long-term adverse effects of the test substance following a repeated administration. In aquatic studies, the test is mainly performed to analyze the water quality and aids in the protection of exposed organisms over the stressors. The test period usually prolongs from 90 days to six months or more, depending on the selection of the test model (Hayes, 2014). Most of the chronic toxicity studies are designed to assess the carcinogenicity of the test chemical as the evaluation period usually lasts for at least 10% of the lifespan of the test organisms, or more than or equal to 6 months of exposure (Hulla *et al*., 2014). Dose selection is an important criterion in chronic toxicity tests, which is usually considered to evaluate certain biological endpoints such as behavior, growth, development, reproduction, transgenerational effects, carcinogenicity, teratogenicity, etc. (Dorato *et al*., 1994).

1.5 Factors affecting toxicity

The toxicity of a chemical substance on the exposed organisms shows variations based on several factors such as physico-chemical

properties of the toxicant, dosage, particularly on the dosage-response and dosage-duration relationships, route of exposure, species exposed, life stages, gender, and age of the species, mode of entry, distribution, metabolism, and excretion of the test substances, health and nutritional status of the organisms, half-life or degradation time of the toxicant, adduct formation of the chemical with other chemicals to form combined or multiple effects, and so on. The aquatic ecosystem offers a unique opportunity to observe the impact of contaminants at various levels as it is the place where all pollutants are dumped. Aquatic organisms are exposed to several toxicants at different levels making the area of aquatic toxicology multidisciplinary (Gupta and Vidyapith, 2019).

The physico-chemical properties of the chemical, particularly the structure are mainly involved in exerting toxic effects, and most of the factors listed above are strictly dependent on the chemical structure. Besides, some of the environmental parameters such as water temperature, pH, salinity, dissolved gases such as oxygen and CO_2 levels, hardness, suspended particles, and dissolved organic matters also influence the toxicity (Howe *et al*., 1994). Certain chemicals are less toxic at high temperatures as the rate of degradation increases with temperature in the natural environment. Similarly, variations in pH and salinity also contribute to influencing the toxicity variations in the exposed organisms (Paul *et al*., 2019).

The toxicity of a chemical is also associated with the bioavailability of pollutants that are determined by the absorption, distribution, and excretion of toxicants. Bioavailability is the presence

of toxicants in absorbable form thereby enhances bioconcentration in test organisms (Howe *et al*., 1994). The water solubility of toxicants is known to affect bioconcentration since the highly soluble substance decreases the chance of bioconcentration because it can easily undergo a detoxification mechanism. The chemicals having a very high octanol-water partitioning coefficient (Kow) are less soluble in water, and highly lipophilic so they are expected to bioconcentrate, and enhance biomagnification on chronic exposure (Teplova *et al*., 2017). The persistence of chemicals is determined by their half-life and rate of degradation in the environment by photodegradation, thermal decay, or microbial decomposition, where the prolonged persistence enhances toxicity (Seyler *et al*., 1994).

The low concentrations of the toxicant exposed for long durations, i.e., chronic studies are found more reliable to evaluate the adverse effects of the chemical substances (Dawson *et al*., 2016; Kim *et al*., 2017). In fish, the mode of entry of the toxicants into the organisms occurs mainly through three routes namely oral, dermal, and gill or operculum. The water-soluble and large particles enter through the oral route, however, small-sized lipophilic compounds penetrate through skin or cross the gill epithelium (Zhang *et al*., 2020; Huang *et al*., 2021). The water partition co-efficient or Kow value determines the affinity of the toxicant towards the lipid bilayer of the organisms. In general, the LogKow value is inversely related to water solubility, and directly proportional to the molecular weight thus lipophilicity or hydrophilicity of a substance is determined. The lipophilic compounds

are hydrophobic, which finds their entry into the organisms orally, or bind with the specific receptors (Kloas *et al*., 2000).

The toxicants are either excreted as the parent compound or its metabolites, where the biotransformation occurs in the liver through phase I and phase II reactions to transform into more hydrophilic compounds and are readily excreted to prevent the adverse effects (Vermeulen, 1996). Biotransformation is a detoxification process, however, bioactivation converts the parent compound into more reactive metabolites causing severe toxic effects (Farrington, 1991). Some chemicals in the natural environment show high affinity to other chemical substances, where the combined effects are found to be more toxic. Besides, age, gender, strain of species, nutritional and health status of species also influence the intensity of the toxicants, which are measured through valuable toxic endpoints such as behavioral modifications, physiological and biochemical analysis, molecular markers, genetic damage, reproductive dysfunctions, carcinogenicity, histopathological changes, etc.

1.6 Behavioral response as an assessment tool

The response of an organism towards the changes in the water quality of their immediate environment is assessed mainly by evaluating their behavior, as it connects the physiological processes with the ecology and environment of an organism. It is a non-invasive approach for hazard identification, and provides an early warning signal for the changes in the environment, diseases, and stressors (Beitinger, 1990). Fish is widely used in studying the behavioral

response, and considered as the sentinels of aquatic ecosystems. The normal fish behavior follows a specific sequence that is triggered by environmental stimuli, which then act through central and peripheral nervous systems. Any changes in the quality of the environment are reflected as modified behavior that ultimately hampers the survival of organisms (Sharma, 2019; Scott and Sloman, 2004). The toxicants bind with the chemosensory receptors of the fish causing irreversible damages to the receptors that subsequently lead to altered behavior (Scott and Sloman, 2004). The failure of receptors also affects several other normal physiological processes that are essential for the survival of the fish (Robinson, 2009).

The most widely monitored behavioral changes in fishes after the toxicant exposure includes variations in swimming pattern, food preferences, foraging, aggression, predatory, avoidance, social and breeding behavior. The behavioral studies were carried out in acute, sub-acute, sub-chronic, or chronic exposure at lethal or sublethal concentrations to check for the water quality (Baatrup, 1991). The fish behavior is a complicated process under the control of the nervous system, and any changes in the behavioral pattern are reflected in the neuronal functions. Besides, changes in the hormones that are related to the stress response also influence the behavior of fish (Munakata and Kobayashi, 2010).

1.7 Histopathology as an endpoint in acute toxicity

Histopathology is a valuable parameter used for the diagnosis and study of diseases, and tissue damages observed under a

microscope. It is widely used in physiology, pathology, and toxicology as a valuable biomarker to assess the response of organisms to chemical stressors (Bozzetta *et al*., 2012). Histopathology is a precise, valid, and reliable tool used for the detection of morphological changes in tissues after acute and chronic toxicant exposure to interpreting the health status of the organism (Ayas *et al*., 2007). Histological examinations are performed in various vital organs such as gills, liver, kidney, spleen, brain, muscles, and gonads to monitor the tissue damage and to evaluate other physiological abnormalities. Histological lesions in gill tissues signify alterations in acid-base balance, ionic regulation, respiration, and excretion of the nitrogenous wastes due to toxicant exposure (Cengiz, 2006).

Modifications in the histology of liver tissues are used as bioindicators for the altered metabolism, as the tissues function with detoxification and biotransformation processes (van der Oost *et al*., 2003). In fish, the head kidney, and spleen are mainly involved in the immune response as they are the crucial sites for antibody production and granulocytopoiesis (Carbone and Faggio, 2016). The pathologies of these tissues denotes altered immune functions thereby leads to the susceptibility of inflammation, infection, and diseases (Martin and Krol, 2017). Toxicant-induced histopathological lesions in the brain tissues are used as a sensitive tool to detect behavioral modifications, lack of orientation and coordination of muscular tones, and imbalance of swimming patterns in fishes. Gonadal histology is widely performed to diagnose the reproductive success of the fish population. It is routinely used as a sensitive biomarker for the identification of sex,

different stages of development, documenting the formation of intersex, the occurrence of gonadal tumors, parasite infections, and other abnormalities (Blazer, 2002).

1.8 Aquatic toxicity of triclosan

Triclosan, a widely used antimicrobial agent possessing an endocrine-disrupting property have been largely detected in the water bodies causing threat to aquatic organisms. Owing to the widespread application, triclosan reaches the non-target organisms, including fish, either directly or indirectly through the food chain. The toxicity of triclosan has been reported in various aquatic organisms including algae, shrimps, snails, fish, and marine mammals (Dann and Hontela, 2011). The analysis of several endpoints has detected pathologies in various aquatic animals associated with the triclosan exposure, which was evident by the altered behavior (Pullaguri *et al*., 2021), growth inhibition (Ma *et al*., 2019), abnormal metabolism (Zhu *et al*., 2018), neuronal dysfunction (Falisse *et al*., 2017), immunosuppression (Bera *et al*., 2020), endocrine disruption (Song *et al*., 2020), and failure of reproduction (Stenzel *et al*., 2019).

1.9 Significance of the study

In this context, the hypothesis of the present study was designed to evaluate the acute toxic effects of triclosan in the freshwater fish, *Anabas testudineus*. In addition, the selection of two main toxicological endpoints such as behavioral modifications and histopathological changes helps to illustrate the toxicity of triclosan.

These endpoints are used as physiological and morphological biomarkers to predict the acute toxicity of triclosan during 96 h exposure period. Based on the estimation of the median lethal concentration, the sensitivity and toxic response of the fish, *A. testudineus* can be studied. The study determines the concentration of triclosan that causes mortality to half of the population within a specific time duration. Further, the estimated lethal concentration can be used as a reference value for the selection of sublethal concentration for long-term toxicity studies.

2. OBJECTIVE OF THE STUDY

- To determine the acute toxicity of triclosan in the fish *Anabas testudineus*

3. REVIEW OF LITERATURE

The United States Environmental Protection Agency (US EPA), in 1997 has approved the use of triclosan as an antimicrobial agent in oral care products, which encouraged the manufacturers to include triclosan as an ingredient in several personal care products for the long-lasting antibacterial protection (Yueh and Tukey, 2016). The biological sewage treatment does not efficiently remove triclosan from the effluents of the wastewater treatment plant, thus their presence has been detected in microgram to nanogram level in all compartments of the aquatic ecosystem (Heidler and Halden, 2007). The toxic effects of triclosan and its metabolites have been documented in various aquatic organisms, including crustaceans, amphibians, and fishes (Rowett *et al*., 2016; Khatikarn *et al*., 2018; Chen *et al*., 2018; Fu and Bae, 2020).

The acute toxicity of triclosan has been demonstrated in various fish species, in which the median lethal concentration or LC_{50} value for 48 h in medaka fry, *Oryzias latipes* has been reported as 352 µg L^{-1} concentration. The lethality to hatchlings has occurred at 1 mg L^{-1} and 500 µg L^{-1} at 24 and 72 h exposure, respectively (Foran *et al*., 2000). The 96 h-LC_{50} values of triclosan in *Pimephales promelas* and *Lepomis macrochirus* have been known to be 260 and 370 µg L^{-1}, respectively. The study has also reported that the no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) of triclosan as 34.1 and 71.3 µg L^{-1}, respectively, which were determined during the early life-stages of *Oncorhynchus mykiss*. In

addition, the average triclosan accumulation factor in *Danio rerio* over the five-week test period has been reported as 4,157 at 3 µg L^{-1}, and 2,532 at 30 µg L^{-1} concentration (Orvos *et al*., 2002).

The fry of *Oryzias latipes* and *Danio rerio* exposed to triclosan for 96 h has illustrated that the median lethal concentration using the probit analysis was 0.40, and 0.22 mg L^{-1}, respectively, along with a reduction in the growth and reproduction of the adult fishes (Tatarazako *et al*., 2004). The effects of triclosan on the early life stages of medaka *Oryzias latipes* has been assessed in 24 h-old larvae, which showed that the median lethal concentration of the toxicant exposed for 96 h was 602 µg L^{-1}. The study has also reported the delayed hatchability of fertilized eggs after triclosan exposure (Ishibashi *et al*., 2004). The acute toxicity study conducted in the fish *Oryzias latipes* after triclosan exposure for 24 h has estimated the median lethal concentration value as 0.60 mg L^{-1} (Kim *et al*., 2009). In another study, the acute toxicity of triclosan has been evaluated in the adult medaka *Oryzias latipes*, which found the median lethal concentration as 1.7 mg L^{-1} when exposed for 96 h in semi-static conditions (Nassef *et al*., 2009).

During the acute toxicity period of triclosan exposure, the behavior of Japanese medaka monitored using the computerized tracking method has been shown to alter the swimming pattern and feeding habits of the fish. Besides, the estimation of NOEC and PEC of triclosan in medaka has been found to be 1.7 and 20 µg L^{-1}, respectively (Nassef *et al*., 2010a). The toxicity of triclosan in the early life-stages of medaka has been illustrated after injecting the eggs of 8 h

post-fertilization, or late blastula stage, which estimated the median effective concentration for their survival as 4.2 ng per egg. Meanwhile, some notable embryonic lesions such as abnormal eye development, hemorrhage, and yolk-sac shrinkage have been observed at the high dose of triclosan (Nassef *et al*., 2010b).

Exposure of triclosan to the zebrafish, *Danio rerio* for 96 h estimated the values of median lethal concentration as 0.42 and 0.34 mg L^{-1} in embryos and adult fish, respectively. The toxicity has been evident by the delayed otolith formation, abnormal eye and body pigmentation, spine malformations, and pericardial edema in the embryos whereas the adult showed alterations in the enzymatic activities, and formation of micronuclei in the peripheral erythrocytes (Oliveira *et al*., 2009). The acute lethality tests of triclosan conducted in 7 d post-hatch larvae of fathead minnows, *Pimephales promelas* have reported the lowest observed effect concentration (LOEC) value for survival as 150 μg L^{-1}, and also resulted in altered swimming and feeding behavior (Fritsch *et al*., 2013).

Another study has reported that the 96 h-LC_{50} value of triclosan in zebrafish was 192 μg L^{-1}, and the observation further revealed that a dose-dependent reduction in the heart rate was found associated with the mortality of the fish (Schmidt *et al*., 2013). The median lethal concentration observed in swordtail fish *Xiphophorus helleri* exposed to triclosan for 96 h was 1.47 mg L^{-1}, however, when the exposure period was increased to 168 h at low concentration demonstrated alterations in phase I and phase II metabolic enzyme activities (Liang *et al*, 2013). In a comparison study, the 96 h-LC_{50} values of four fish

species namely *Pseudorasbora parva* (71 µg L^{-1}), *Carassius auratus* (1.839 mg L^{-1}), *Misgurnus anguillicaudatus* (45 µg L^{-1}), and *Tanichthys albonubes* (0.889 mg L^{-1}) illustrated that the demersal fish *M. anguillicaudatus* was the most sensitive species (Wang *et al*., 2013).

Embryos of zebrafish exposed to triclosan at different concentrations during 8 to 120 h post-fertilization stage under a static condition have been shown to cause mortality to half of the test population at 406 µg L^{-1} along with the reduction in development and hatching success thereby proved the developmental toxicity (Carmosini *et al*., 2016). The median lethal concentration determined in the freshwater fish, *Oreochromis niloticus* using probit analysis has been found to be 2.81 mg L^{-1}, which showed a dose-dependent increase in mortality with prominent behavioral modifications in the form of mucus deposition and lethargy during the exposure period (Vijitha *et al*., 2017). A study conducted in the female Yellow River carp, *Cyprinus carpio* under the semi-static condition has reported that the 96 h-LC_{50} value of triclosan was 0.80 mg L^{-1}, along with behavioral alterations such as spiral movement along the walls of the aquarium, upward swimming to gulping air, lethargic and bottom settlement until the death has been recorded (Wang *et al*., 2017).

In an avoidance test, the fish *Poecilia reticulata* has been allowed for forced and non-forced exposure systems to identify the potential effects of triclosan on spatial displacement and local population decline. The study demonstrated that the fish avoided triclosan in a non-forced environment thereby reduced the rate of mortality. The population immediate decline observed by the

avoidance response revealed that the fish moved towards the favorable environment to escape from triclosan toxicity by spatial displacement mechanism. This confirmed that triclosan is one of the potential environmental disturbers that decline the fish population in the natural environment (Silva *et al*., 2017).

A study on the acute toxicity tests in the adult *Pangasianodon hypophthalmus* had reported 1458 μg L^{-1} as the LC_{50} value, and the treatment-related behavioral responses such as air gulping, escaping or jumping tendency, hyperventilation, respiratory distress, sluggish movement, and uncoordinated swimming (Sahu *et al*., 2018). The acute toxicity of triclosan in the freshwater fish *Channa striatus* estimated after 96 h has been found to be 0.602 ppm, along with behavioral modifications such as fast air gulping, lethargic, and abnormal swimming pattern (Mary and Jose, 2018).

Goldfish, *Carassius auratus* exposed to triclosan at six different concentrations such as 0.6000, 0.6900, 0.7935, 0.9125, 1.0494, and 1.2068 mg L^{-1} for 96 h has found that 1.0496 mg L^{-1} concentration as the LC_{50} value. Besides, during the period some behavioral abnormalities like rapid opercular movement, loss of balance, lethargy, and bottom settlement in the tank have been reported with an increase in triclosan concentration (Wang *et al*., 2018). The combined exposure of triclosan, 2,4-dichlorophenol and 2,4,6-trichlorophenol to zebrafish *Danio rerio* in the ratio of 1:2:4 has found LC_{50} and EC_{50} values of the mixtures as 2.28 and 1.16 mg L^{-1}, respectively with delayed hatching rate, developmental defects, and

dysfunction in cardiovascular circulation, and defective fat metabolism (Zhang *et al*., 2018).

A static renewal acute toxicity test conducted in an Indian major carp, *Catla catla* has observed 0.36 mg L^{-1} as the median lethal concentration, and the 95% confidence intervals ranged between 0.348 and 0.379 mg L^{-1} thereby revealed that triclosan was highly toxic to the exposed fish (Hemalatha *et al*., 2019). The median lethal concentration of triclosan in the early life stages of four food fishes namely *Cyprinus carpio*, *Ctenopharyngodon idella*, *Labeo rohita*, and *Cirrhinus mrigala* has been found to be 0.315, 0.116, 0.096, and 0.131 mg L^{-1}, respectively. In addition, the study also reported a concentration-dependent delay in embryonic development, delayed hatching rate, spine malformations, edema, and deflated swim bladder, and reduction in length and weight of hatchlings along with an increase in abnormal embryos (Dar *et al*., 2019).

In a static bioassay test, the median lethal concentration of triclosan for 24 h has been observed as 0.9 mg L^{-1} and displayed behavioral changes such as circular movements, erratic and fast swimming around the tank, backward and jerking movement in the fish *Labeo rohita* (Geetha and Priya, 2020). The median lethal concentration of triclosan has been carried out at different pH under the static renewal system using the probit analysis method, which demonstrated 0.91, 1.11, and 1.38 mg L^{-1} as 96 h-LC_{50} values at pH 6.5, 7.5, and 8.5, respectively in the juvenile fish *Pangasianodon hypophthalmus* thereby illustrated that the acute toxicity of triclosan was negatively correlated with pH of water (Paul *et al*., 2020b).

Similarly, increase in temperature from 25 to 35 ºC has been shown to decrease the acute toxicity in *Pangasianodon hypophthalmus* (Paul *et al*., 2020b). In the adult male zebrafish, the 96 h-LC_{50} value, with a 95% confidence interval within the range of 389.2–415.3 μg L^{-1} has been reported as 398.9 μg L^{-1} (Gyimah *et al*., 2020). A recent study has estimated the median lethal concentration of triclosan for 96 h in zebrafish embryos as 0.6 mg L^{-1} thereby caused aberrations in motor neuron innervations in skeletal muscles, and reduced touch-evoked escape response in zebrafish larvae (Pullaguri *et al*., 2021).

4. MATERIALS AND METHODS

4.1 Test animal

Anabas testudineus (7 ± 1 g; 7 ± 1.5 cm) were collected from the Pulimugham Aquafarms and hatcheries, Alappuzha district, Kerala, India. The care and use of the fish complied with the Animal Welfare Board of India and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the Ministry of Environment, Forest, and Climate change, Government of India. Fish were transported to the laboratory and acclimatized for two weeks in dechlorinated, aerated, semi-static water in glass tanks of 40 L capacity (30 cm width × 60 cm length × 30 cm depth) at 12 h light: 12 h dark photoperiod.

4.2 Chemicals

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) of 97% purity, was purchased from HiMedia Research Laboratories, Mumbai, India. Dimethyl sulfoxide (DMSO; ≥99% purity), haematoxylin, eosin, xylene, DPX mountant, and all other chemicals used in the present study were of analytical grade and purchased from the local commercial sources.

4.3 Acute toxicity test

After acclimatization, based on the previous preliminary experiments, fish were exposed to seven different concentrations of

triclosan i.e., 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, and 2.2 mg L^{-1} for 96 h along with negative control and solvent-control (DMSO) groups, maintaining 10 animals in each group. Test concentrations were chosen in the range after repeated range-finding experiments. Static test condition was maintained in the experiment for the determination of median lethal concentration. Other disturbances that affect the behaviour of the fish were avoided to monitor behavioural changes after triclosan exposure, which were inspected for 2 to 3 h immediately after the toxicant exposure, and regularly for 1 h continuously at every 24 h interval (Eissa *et al*., 2010), and scoring pattern was followed as described by Sahu *et al.* (2018). The mortality of fish in each treatment group was recorded throughout the experimental period. Median lethal concentration (LC_{50}-96 h) that kills 50% of the test animal within 96 h was plotted against different concentrations of triclosan on MS-Excel. The values are then computed with P=0.95 confidence limits by using probit analysis (Finney, 1971).

4.4 Body weight and mucus deposition

After the treatment period, fish captured from each group were weighed and recorded. Then fish weights were again recorded without mucus as described by Al-Rasheed *et al* (2018) with minor modifications. Briefly, a sterile glass slide was used to carefully scrape the mucus from the body of the fish dorsolaterally, starting from the head sliding towards the caudal region. Care was taken to avoid pressure on the epithelial cells or scales as well as on the ventral vent portion to prevent urogenital contamination. The difference between

the weights of fish with and without mucus expressed in percentage provides the rate of mucous deposition.

4.5 Collection of tissues

In the acute toxicity tests, triclosan exposed at 1.6 and 1.8 mg L^{-1} concentrations showed 40 and 60% mortality, respectively, between which the median lethal concentration was expected to occur. The weights of gill and liver tissues were recorded from the above concentration groups. Histopathological analysis were performed in the gill and liver tissues obtained from 1.6 and 1.8 mg L^{-1} concentrations of triclosan-exposed groups, and compared with the respective control tissues.

4.6 Histopathological analysis

Tissues were fixed in 10% neutral buffered formalin for 24 h and dehydrated in series of ascending grades of alcohol, then tissues were cleared in xylene until they became translucent. The tissues were embedded in molten paraffin wax for an hour for the complete impregnation to make the tissue blocks. Sections were made using rotary microtome at 4 to 6 μm thickness, which was double stained with hematoxylin and eosin, and finally mounted in DPX mountant (Roberts and Smail, 2001). Triplicate slides of gill and liver tissues were examined under the light microscope at 400X magnifications. Photomicrographs were taken using a canon shot camera fitted to the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope. The semi-quantitative scoring of tissue lesions were represented in Tables

and the grading system was adopted as described by Peebua *et al*. (2006) with slight modifications.

4.7 Statistical analyses

Statistical analyses were performed using International Business Machine Corporation- Statistical Package for the Social Sciences (IBM-SPSS) software, version 17.0. The total number of animals used in the experiments, the test concentrations, and percentage of mortality observed in each experimental group were fit to a probit analysis using log10 concentration transformation.

5. RESULTS

5.1 Acute toxicity or median lethal concentration (LC_{50}-96 h) of triclosan in the fish, *Anabas testudineus*

Acute toxicity of triclosan was determined in the freshwater fish *Anabas testudineus* for 96 h using probit analysis. During the experimental period, fish from the control groups were active and healthy without any mortality. Exposure of triclosan at seven different concentrations such as 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, and 2.2 mg L^{-1} were the treatment groups, in which the toxicant exposed at 1 mg L^{-1} for 96 h showed no mortality (Table 1). The mortality of fish was confirmed by unresponsive conditions on touching the caudal peduncle using glass rods and without opercular movement. Fish exposed to triclosan at 1.2, 1.4, 1.6, 1.8 and 2.0 mg L^{-1} concentrations showed 10, 30, 40, 60, and 70 % mortality, respectively (Table 1). In the 2.2 mg L^{-1} exposure group, 100 % mortality of the fish was observed within 96 h. The dead fish observed during the study were immediately removed from the experimental tank to avoid contamination, and the mortality was recorded for probit analysis.

The observations showed an increase in the percentage of mortality for an increase in the concentration of triclosan. The recorded percentage of fish mortality was used for correlation analysis between different concentrations of triclosan, which showed a high degree of positive co-efficient of correlation i.e., r = +0.869. The mortality percentage (Y variable) and concentrations (X variable) plotted on the

graph using MS Excel obtained the linear regression equation as y = 80.35x - 84.28, and coefficient of determination, R^2 = 0.981. The median lethal concentration of triclosan for 96 h (LC_{50}-96 h) at 95% confidence limits using probit analysis was determined as 1.767 mg L^{-1} in the fish *Anabas testudineus* (Table 2).

5.2 Behavioural changes

Fish exposed to triclosan at seven different concentrations for 96 h showed alterations in the normal behavioral pattern when compared with the control fishes. The severity of changes in the behavioral responses increased with an increase in the concentrations of the toxicant, which was shown in Table 3. Fish when introduced to the toxicant, above 1.2 mg L^{-1} concentrations, showed an immediate response like jumping or escaping out from the experimental tanks, semi-circular swimming, and scoliosis. Later after some time, fish exhibited jerky vertical movement and surface swimming along with an increase in the mucous deposition throughout the body surface. The tendency of the fish to engulp air to avoid the uptake of toxicant was more prominent, followed by erratic swimming that resulted in frequent knocking at the walls of tanks, and also among themselves.

At the time of mortality, fish showed initial loss of equilibrium where the surface swimming pattern changed to bottom swimming by mostly settling at the corner of the tanks with less movement. It was then followed by reduced opercular movement, and fish became slowly lethargic where the dead fishes are found with protruded eyes, high mucous deposition, and locked jaws. In comparison, fishes from

the control groups when introduced into new tanks for the first time showed initial stress as evident by its slow movement and gathering at the corner of the tank. Later within an hour, they explored the tank without any stress and showed no abnormal behavior as observed in the treatment groups. Control fishes were healthy and active throughout the experimental period, and no mortality was observed.

5.3 Body weight, mucus secretion, and tissue weights

The body weight of the fish measured before and after triclosan exposure showed no significant changes when compared with the corresponding control groups. The data for the two concentrations of triclosan-exposed groups namely 1.6 and 1.8 mg L^{-1} alone were shown in Fig. 1. However, the mucus deposition increased significantly ($P<0.05$) in the above concentration groups of triclosan treatment than the respective control groups (Fig. 2). A significant ($P<0.05$) reduction in the weights of liver and gill tissues was observed after 1.6 and 1.8 mg L^{-1} concentration groups of triclosan, when compared with the corresponding control tissues (Fig. 3).

5.4 Histological changes

Histological analysis of gill and liver tissues was also performed at two concentrations of triclosan (1.6 and 1.8 mg L^{-1} concentrations) and was compared with the respective control tissues. The pathologies observed after triclosan exposure was scored, and listed in Table 4. Gill tissues from the control groups showed normal gill arches, prominent gill epithelium, and well-organized primary and

secondary lamella (Fig. 4). Some of the histological alterations observed in the gill tissues of triclosan-exposed groups include aneurysm, upliftment of gill epithelium, hyperplasia and lamellar disorganization, fusion, and loss of secondary lamellae (Fig. 4). Control liver tissues showed normal hexagonal hepatocytes with homogenous cytoplasm, and central spherical nucleus (Fig. 5). Triclosan-exposed liver tissues showed several modifications in the histoarchitecture like anucleated or spindle-shaped nucleus, degenerated hepatocytes, melanomacrophage aggregation, and cytoplasmic vacuolization (Fig. 5).

Table 1 Mortality rate of the fish, *Anabas testudineus* exposed to triclosan for 96 h (n = 10/ group, in replicates)

Concentration (mg L^{-1})	Mortality (No. of animals)	Mortality (%)	Hour of mortality
Negative control	0	0	96 h
Vehicle control (DMSO)	0	0	96 h
1	0	0	96 h
1.2	1	10	96 h
1.4	3	30	96 h
1.6	4	40	96 h
1.8	6	60	96 h
2.0	7	70	96 h
2.2	10	100	96 h

Table 2 **Probit analysis showing LC_{50}-96 h of triclosan in *Anabas testudineus***

Prob.	Concentration	95% Confidence Limits	
		Lower	Upper
.01	1.18286	.69123	1.40558
.02	1.23985	.76485	1.45247
.03	1.27743	.81534	1.48346
.04	1.30645	.85535	1.50749
.05	1.33053	.88921	1.52754
.06	1.35139	.91899	1.54498
.07	1.36994	.94582	1.56060
.08	1.38676	.97042	1.57485
.09	1.40225	.99327	1.58804
.10	1.41665	1.01471	1.60041
.15	1.47788	1.10752	1.65410
.20	1.52843	1.18570	1.70034
.25	1.57317	1.25551	1.74334
.30	1.61446	1.31987	1.78533
.35	1.65369	1.38038	1.82787
.40	1.69180	1.43799	1.87228
.45	1.72951	1.49325	1.91982
.50	**1.76743**	**1.54652**	**1.97183**
.55	1.80619	1.59809	2.02981
.60	1.84645	1.64826	2.09554
.65	1.88900	1.69747	2.17120
.70	1.93490	1.74640	2.25972
.75	1.98569	1.79614	2.36542
.80	2.04381	1.84839	2.49540
.85	2.11372	1.90616	2.66302
.90	2.20508	1.97565	2.89844
.91	2.22773	1.99208	2.95943
.92	2.25260	2.00982	3.02755
.93	2.28027	2.02922	3.10471
.94	2.31157	2.05079	3.19370
.95	2.34780	2.07529	3.29892
.96	2.39109	2.10400	3.42769
.97	2.44540	2.13927	3.59388
.98	2.51951	2.18625	3.82880
.99	2.64091	2.26083	4.23352

Table 3 **Behavioral modifications in the fish *Anabas testudineus* exposed to triclosan for 96 h (n=10/ group, in replicates)**

Behavioral parameters	Concentration (mg L^{-1})								
	Control	**DMSO**	**1**	**1.2**	**1.4**	**1.6**	**1.8**	**2**	**2.2**
Erratic Swimming	-	-	-	-	-	+	+++	++++	++++
Loss of equilibrium	-	-	-	+	++	+++	++++	++++	++++
Reduced opercular movement	-	-	+++	+++	++	+	+	+	+
Frequent air gulping and surfacing	-	-	+	+	++	+++	++++	++++	++++
Increased mucous deposition	-	-	+	+	++	++	+++	++++	++++
Escaping or jumping tendency*	-	-	-	+	+	++	+++	++++	++++
Semi-circular swimming behavior*	-	-	-	-	-	+	+++	++++	++++
Scoliosis*	-	-	-	-	-	+	+++	++++	++++

Signs denotes - None, + Mild, ++ Moderate, +++ Strong, ++++Very strong

*** Symptom observed initially at higher concentrations only**

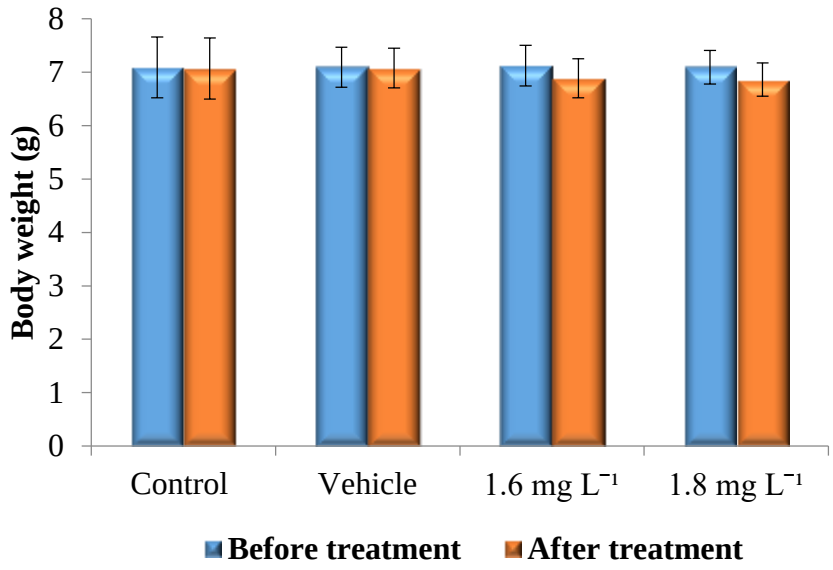

Fig. 1 Effect of triclosan on the body weight of *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates)

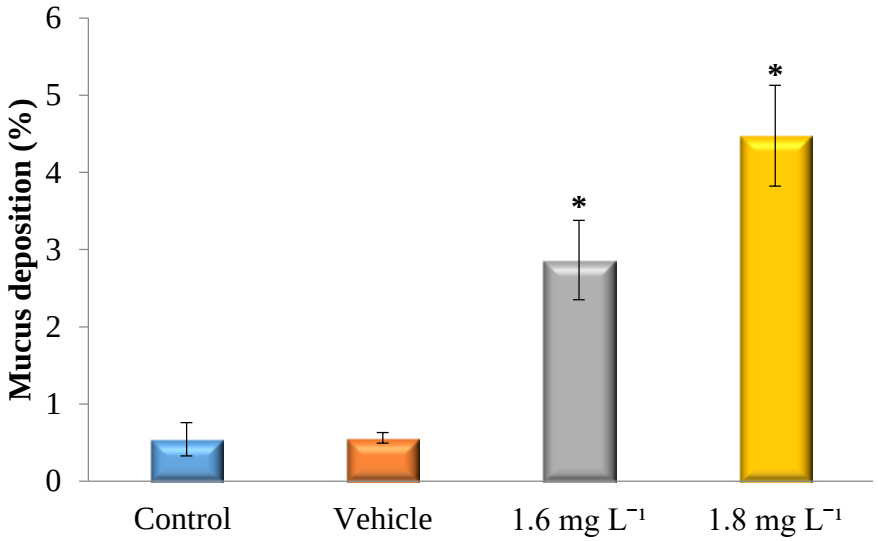

Fig. 2 Effect of triclosan on mucus deposition in the fish, *Anabas testudineus* (Mean ± SD; Asterisks (*) denotes significance at P<0.05 against the control groups; n = 10/ group, in replicates)

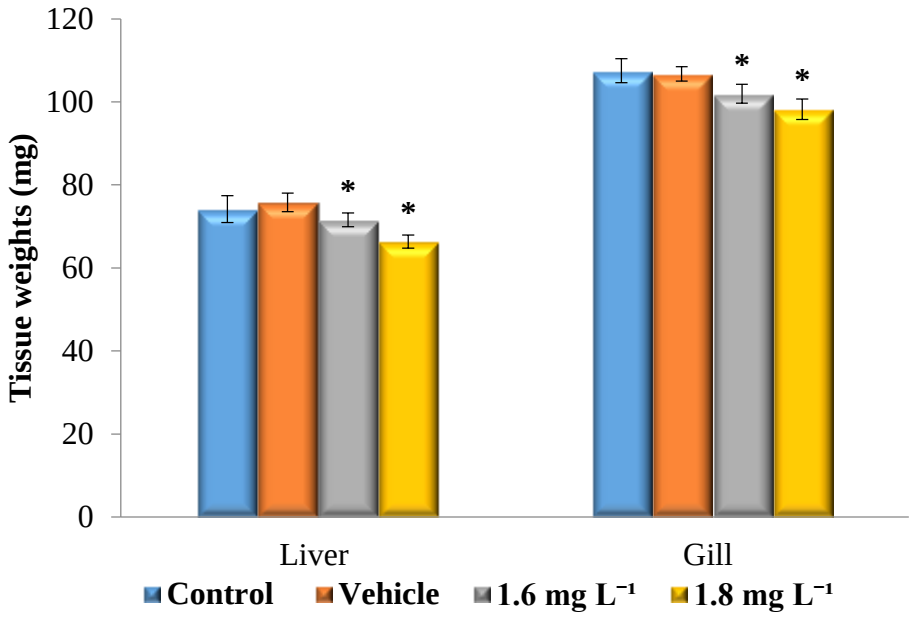

Fig. 3 Effects of triclosan on tissue weights in the fish *Anabas testudineus* (Mean ± SD; Asterisks (*) denotes significance at P<0.05 against the control groups; n = 10/ group, in replicates)

Table 4 **Semi-quantitative scoring showing histopathological lesions in the gill and liver tissues of *Anabas testudineus* after triclosan exposure (n=10/ group, in replicates)**

Histopathological lesions	**Concentration (mg L^{-1})**			
Gill tissue	**Control**	**Vehicle**	**1.6**	**1.8**
Aneurysm	_	_	++	+++
Upliftment of gill epithelium	_	_	++	+++
Lamellar disorganization	_	_	++	+++
Fusion or loss of secondary lamellae	_	_	++	+++
Hyperplasia of interlamellar epithelium	_	_	++	+++
Liver tissue	**Control**	**Vehicle**	**1.6**	**1.8**
Cytoplasmic vacuolization	_	_	+++	+++
Degenerated cytoplasm	_	_	+++	+++
Melanomacrophage aggregation	_	_	++	+++
Anucleated hepatocytes	_	_	++	+++
Spindle-shaped nucleus	_	_	++	+++

Signs denotes - None, + Mild, ++ Moderate, +++ Strong alterations

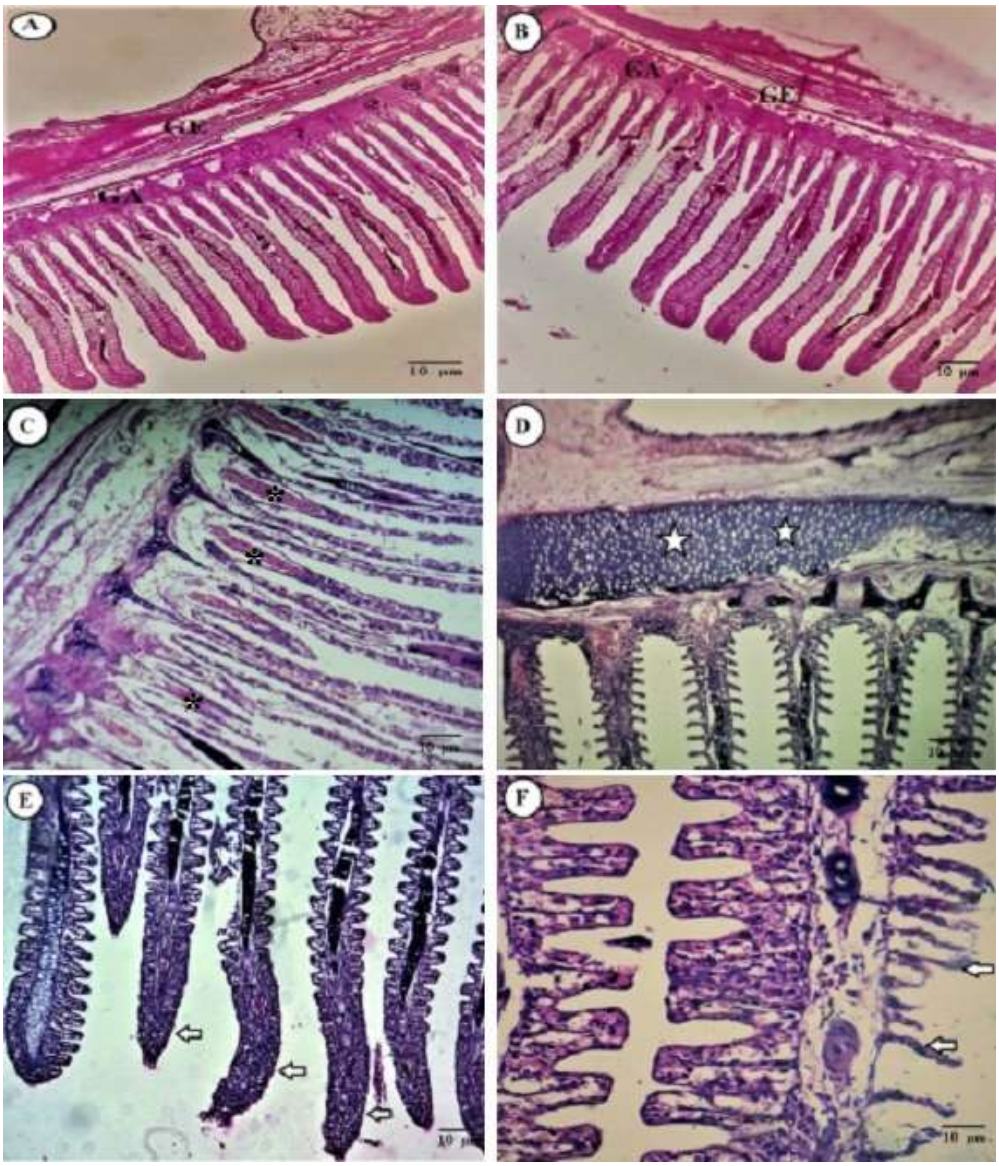

Fig. 4 Histopathology of gill tissue in the fish, *Anabas testudineus* **A:** Control tissue; **B:** Vehicle control tissue (DMSO); **C-F:** Triclosan-exposed groups. GE- Gill epithelium, GA-Gill arches, Asterisks (black)-Aneurysm, Asterisks (white)-Mucous deposition, White arrows- Fusion or loss of secondary lamella

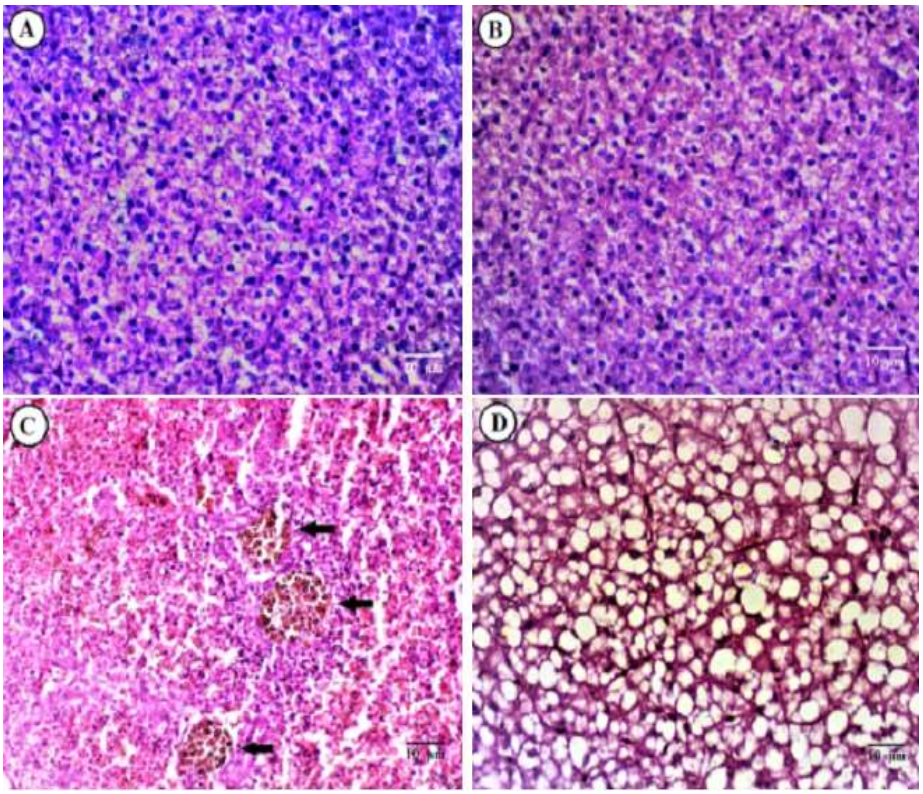

Fig. 5 Histopathology of liver tissue in the fish, *Anabas testudineus*
A - Control tissue; **B** - Vehicle control tissue (DMSO); **C** - Triclosan at 1.6 mg L^{-1} exposed for 96 h showing Melanomacrophage aggregation (arrows); **D** - Triclosan at 1.8 mg L^{-1} exposed for 96 h showing Vacuolization

6. DISCUSSION

6.1 Median lethal concentration (LC_{50}-96 h) of triclosan

In aquatic toxicology, acute toxicity tests provide the basic information on the safety evaluation of chemicals that enters intentionally or unintentionally into the aquatic ecosystem. The comparison of new compounds with structurally or biologically-related substances can be evaluated by choosing the critical dosage range, like the lowest dose at which the possible adverse effects occur, and the highest dose without such effects. Besides, the signs of abnormal responses in the form of behavioral modifications, alterations in physiological and metabolic functions, mortality, and histopathological changes in the target organs provide the probable mode of action of the test substances (Arome and Chinedu, 2014).

The use of triclosan, an antimicrobial agent, in topical administration (0.3%), oral medication (0.03%), and as an additive in plastic industries (1 to 10%) has been approved in some countries. Thus it enters into the consumer markets globally, and the estimated amount of discharge into the wastewater was approximately 1.1×10^5 kg $year^{-1}$ (Olaniyan *et al*., 2016). In ecotoxicology, the risk assessment of the test chemical is evaluated based on the acute toxicity tests, which refers to the study of the adverse effects of a chemical after a single dose within a short time interval. The current study analyzed the median lethal concentration of triclosan in the freshwater fish *A. testudineus* on exposure to seven different concentrations for 96 h.

According to OECD guidelines of acute toxicity tests, the preliminary range-finding tests were conducted before the selection of concentrations (OECD, 1992). *A. testudineus* collected from the Alappuzha site was selected concerning age, sex, length, weight, and physiological status for the uniformity, and were acclimatized under constant environmental conditions such as normal range of light, temperature, humidity, housing, and access to food and water. The mortality of fish during the exposure period from every concentration group was confirmed by touching the caudal peduncle using glass rods. Fish found in unresponsive conditions without opercular movement was considered dead and were immediately removed from the treatment tanks to avoid contamination.

Fish exposed to triclosan at 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, and 2.2 mg L^{-1} concentrations showed 0, 10, 30, 40, 60, 70, and 100% mortality, respectively. The current study observed the median lethal concentration of triclosan for 96 h (LC_{50}-96 h) as 1.767 mg L^{-1} in the fish *A. testudineus.* The LC_{50}-96 h of triclosan has been documented in different fish species such as in the rainbow trout *Oncorhynchus mykiss* was 4.4 mg L^{-1} (Adolfsson-Erici *et al*., 2002), fathead minnow *Pimephales promelas* was 0.26 mg L^{-1}, sunfish *Lepomis macrochirus* was 0.37 mg L^{-1} (Orvos *et al*., 2002), medaka *Oryzias latipes* was 0.37 to 1.7 mg L^{-1} (Orvos *et al*., 2002; Nassef *et al*., 2009), zebrafish *Danio rerio* was 0.34 mg L^{-1} (Oliveira *et al*., 2009), Swordtail *Xiphophorus helleri* was 1.47 mg L^{-1} (Liang *et al*., 2013), Nile tilapia *Oreochromis niloticus* was 2.81 mg L^{-1} (Vijitha *et al*., 2017), *Pangasianodon hypophthalmus* was 1.458 mg L^{-1} (Sahu *et al*., 2018), and Indian major

carp *Catla catla* was 0.36 mg L^{-1} (Hemalatha *et al*., 2019). The variations in 96-h LC_{50} values in fish species could be due to differences in age, sex, species difference, sensitivity, mode of exposure, detoxifying mechanisms, and adaptation capacity of the test animal.

6.2 Effects of triclosan on behavioral modifications in the fish

Behaviour is the adjustment of an organism to external and internal stimuli to adapt constantly to the changes in physical, chemical, biological, and social aspects of the environment (Little and Brewer, 2001). Fish behavior is a complicated subject evaluated for ecological risk assessments since it forms a link between the nervous system and the ecosystem. The major factors that influence fish behavior include water temperature, sex ratio, stocking density, reproductive status, and biological rhythm (Lall and Tibbetts, 2009). Besides, exposure to various stressors or toxic chemicals directly or indirectly influences the behavior of fish. Feeding, swimming, and avoidance behavior are the major parameters used to assess the physiological status of the species exposed to any contaminants (Robinson, 2009).

Exposure of triclosan at different concentrations to the fish *A. testudineus* resulted in severe behavioral modifications including aggressive swimming, loss of equilibrium, reduced opercular movement, frequent air gulping, surfacing, increased mucus deposition, escaping or jumping tendency, semi-circular swimming, and scoliosis. Preference to stay at the top layer of the glass tank may be due to the increase in demand for oxygen to withstand the respiratory stress

caused by triclosan treatment. Aggressive behavior was more prominent in the initial period of triclosan exposure, and this could be a self-defensive mechanism to escape from the toxicant. Similar aggressive behavior has been observed after the exposure of tributyltin in the adult zebrafish, *Danio rerio* (Liu *et al*., 2020).

The behavioral changes such as anxiety and loss of equilibrium after triclosan exposure have been found associated with a decrease in the activity of acetylcholinesterase enzyme, and down-regulation of myelin basic protein (*mbp*) and synapsin IIa (*syn2a*) gene expression in the brain and skeletal muscle tissues of adult zebrafish (Pullaguri *et al*., 2020). Besides, prolonged exposure to triclosan has been shown to alter structure and function of the motor neurons in zebrafish embryos that reduced touch-evoked escape response along with down-regulation of acetylcholinesterase, *mbp*, and *syn2a* gene expressions (Pullaguri *et al*., 2021). In the present study, after immediate exposure to triclosan, fish showed some of the notable behavioral changes such as semi-circular swimming, escaping or jumping tendency, loss of equilibrium like vertical hanging, and backward swimming in all treatment groups, regardless of concentrations.

Frequent gulping of air was observed in triclosan-exposed groups along with surfacing behavior, and this could be an attempt of the fish to escape from the toxicant. A similar observation has been reported after long-term exposure of fullerene C_{60} carbon nanomaterial in the fish, *Anabas testudineus* (Sumi and Chitra, 2019). The current study observed lethargy with an increase in triclosan concentration, where the fish remained static at the bottom of the tank for the

remaining exposure period. Reduction in the swimming activities in *A. testudineus* was found consistent with the rainbow trout, *Oncorhynchus mykiss* exposed to triclosan for 61 d (Orvos *et al*., 2002). The decreased opercular movement observed after triclosan exposure denotes respiratory distress, and this could lead to failure of oxygen uptake causing fish mortality (Asifa and Chitra, 2015; Asifa *et al*., 2016).

6.3 Effects of triclosan on body weight, mucus deposition, and tissue weights

Exposure to triclosan did not cause any significant changes in the body weight of the fish, however, the mucus production increased 6 to 8-times in the treatment groups when compared with the control groups, and this could be probably due to irritation of the skin against the toxicant exposure. Moreover, the results suggested that exposure of the triclosan could have stimulated the goblet cells of gill epithelium for mucus secretion thereby forming a thick deposition throughout the body of fish to prevent the entry of toxicant to some extent. Another study also reported similar observations in the freshwater fish, *Oreochromis niloticus* after triclosan exposure (Vijitha *et al*., 2017). In toxicity studies, the tissue weights were recorded to monitor the tissue damage after the toxicant exposure. The current study observed a reduction in the weights of gill and liver tissues, which indicated that triclosan targeted multiple tissues, which was found similar to silicon dioxide nanoparticles exposure in the fish, *Oreochromis mossambicus* (Vidya and Chitra, 2018a).

6.4 Effects of triclosan on histology of gill and liver tissues

Histology is the sensitive tool used in toxicology for the identification and interpretation of toxicant-induced tissue damages and also serves as an indicator of fish health. The severity of tissue damage depends on several factors such as the nature of the toxicant, its concentration, mode of exposure, and the duration of exposure along with the sensitivity of organisms exposed. Gill and liver tissues are considered the most targeted tissues due to toxicant exposure. Gill tissue is the primary organ found in direct contact with an immediate environment so any change in the water quality is reflected in the tissue. Meanwhile, the liver is the organ for detoxification, and the primary site for metabolic, endocrine, and secretory functions. Hence histopathological examination of both gill and liver tissues provides reliable endpoints to determine the adverse effects of triclosan. In the present study, histological analysis revealed that triclosan exposure at 1.6 and 1.8 mg L^{-1} concentrations caused severe morphological lesions in gill and liver tissues of the freshwater fish, *A. testudineus*.

Gill tissue is the major respiratory organ in fish and is also involved in ion regulation, osmoregulation, ammonia excretion, acid-base balance, etc (Dymowska *et al*., 2012). The major histological alterations observed in gill tissues after triclosan exposure includes aneurysm, upliftment of gill epithelium, hyperplasia, lamellar disorganization, fusion, and loss of secondary lamellae. The aneurysm is always related to irreversible pathology in fish gills formed by the collapse of the pillar cell system that affects the vascular integrity, and lamellar epithelial disruptions thereby cause hemorrhage in gill tissue

(Sales *et al*., 2017). The upliftment of gill epithelium observed after triclosan exposure indicates that the structural modifications of the tissue occurred in response to the toxicant exposure. The gill lesions contained epithelial lifting has been reported in the mosquitofish, *Gambusia affinis* after exposure to sublethal concentration of triclosan (Song *et al*., 2021).

Hyperplasia is the ample proliferation of certain cell types such as mucus, epithelial, and chloride cells in the gill lamellae to overcome various stress conditions (Strzyzewska, 2016). Chloride cell proliferation also regulates the exchange of normal ionic balance and reduces the respiratory and excretory surface of the gills that ultimately impairs their normal functions (Strzyzewska-Worotynska *et al*., 2017). Hyperplasia may also increase the epithelial thickness that hinders the normal flow of blood, and leads to the fusion of gill lamellae (Strzyzewska *et al*., 2016). In the present study, disorganized and shortened primary lamellae, fusion, and loss of secondary lamellae were observed after triclosan exposure, which may lead to respiratory distress causing mortality of the fish (Kasherwani *et al*., 2009; Kaur and Dua, 2015). Similar pathological modifications in gill tissues have been documented after chlordecone and chlorpyrifos exposure in the fish *Pseudetroplus maculatus* (Asifa and Chitra, 2017; Raibeemol and Chitra, 2016), and after silicon dioxide and aluminum oxide nanoparticles exposure in *Oreochromis mossambicus* (Vidya and Chitra, 2018a; 2018b). Thus histopathological alterations found in the gill tissues after triclosan exposure may be used as a useful indicator of environmental pollution.

The liver is the organ typically associated with detoxification and biotransformation of toxicants, and hence more susceptible target tissue. In the present study, triclosan caused severe histomorphological changes such as cytoplasmic vacuolization, anucleated or spindle-shaped nucleus, degenerated hepatocytes, and melanomacrophage aggregation. Vacuolar cytoplasm of the hepatocytes contains lipids and glycogen granules, which are related to the normal metabolic functions of the liver, however, a large vacuole formation denotes lipid dystrophy (Abdel-Moneim *et al*., 2012), protein synthesis inhibition, microtubule disaggregation, and energy depletion (Hinton and Lauren, 1990; Younis *et al*., 2013).

Vacuole formation is the cellular defense mechanism, which prevents injurious substances to interfere with the normal biological activities of cells. Vacuolization prevents the synthesis and release of cellular products into the circulation thereby alters normal metabolic functions. Similar hepatic lesions have been reported after sublethal exposure of triclosan in the mosquitofish, *Gambusia affinis* (Song *et al*., 2021). In the present study, spindle-shaped as well as anucleated hepatocytes along with hepatic degeneration suggests metabolic dysfunction. Besides, the formation of melanomacrophage centers, in the form of yellow-brown granules in the hepatocytes denotes the humoral adaptive immune response against the toxicant (Agius and Roberts, 2003). Similar histopathological effects have been observed in the rainbow trout after exposure to a mixture of antimicrobial agents such as triclosan, chloroxylenol, methylisothiazolinone, and borax (Capkin *et al*., 2017).

7. CONCLUSIONS

1. The median lethal concentration of triclosan in the freshwater fish, *Anabas testudineus* evaluated using probit analysis was 1.767 mg L^{-1} concentration.
2. The abnormal behavioral pattern in the fish after triclosan exposure indicates deterioration of water quality.
3. An increase in the mucus deposition after triclosan treatment revealed the primary defensive mechanism of the fish against the toxicant.
4. Exposure to triclosan caused histological changes in gill tissues as evident by an aneurysm, upliftment of gill epithelium, hyperplasia, and lamellar disorganization, fusion, and loss of secondary lamellae, thereby indicated severe respiratory stress.
5. Triclosan exposure caused hepatic lesions such as cytoplasmic vacuolization, anucleated or spindle-shaped nucleus, degenerated hepatocytes, and melanomacrophage aggregation, which may impair the metabolic functions in the fish.

8. REFERENCES

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CHAPTER 4

Chronic effect of triclosan on the gonadal development in the pre-spawning phase of *Anabas testudineus*

Chapter 4 Chronic effect of triclosan on the gonadal development in the pre-spawning phase of *Anabas testudineus*

CONTENTS

LIST OF ABBREVIATIONS

°C	Degree Celsius
µg L^{-1}	Microgram per Litre
µm	Micrometer
µM	Micromol
17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
ANOVA	Analysis of Variance
APHA	The American Public Health Association
cDNA	Complementary deoxyribonucleic acid
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CT	Cycle threshold
cyp19a1a	Cytochrome P450, family 19, subfamily a1a gene
cyp19a1b	cytochrome P450, family 19, subfamily a1b gene
d	days
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DPX	Dibutylphthalate Polystyrene Xylene
E2	Estradiol
ELISA	Enzyme-linked immunoassay
esr1	Estrogen receptor alpha gene
esr2	Estrogen receptor beta gene
foxl2	Forkhead transcription factor gene
FSH	Follicle stimulating hormone
fshr	Follicle-stimulating hormone receptor gene
g	Relative centrifugal force
gapdh	Glyceraldehyde 3-phosphate dehydrogenase gene

GSI	Gonadosomatic index
L	Litre
LH	Luteinising hormone
lhr	Luteinizing hormone receptor gene
mg	Milligram
mg L^{-1}	Milligram per litre
min	Minutes
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
ng	Nanogram
nm	Nanometer
OECD	The Organisation for Economic Co-operation and Development
PPCPs	Pharmaceuticals and personal care products
ppm	Parts per million
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
rpm	Rotation per minutes
StAR	Steroidogenic acute regulatory gene
USA	United States of America
v/v	Volume per volume
vtg	Vitellogenin gene

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1. INTRODUCTION

1.1 Gonadal steroidogenesis in teleost fish

In teleost fishes, several steroidogenic enzymes, sex steroid hormones, and steroid metabolites of gonadal origin have been detected, but only a few are identified to be involved in various reproductive functions. Steroidal hormones and their metabolites of fishes are released into the surrounding water, where they act as a sex pheromone to attract the opposite sex, and function as modulators to maintain sexual behavior. Teleost fish synthesizes several types of bioactive steroids in the specialized cells within the gonadal tissues like theca and granulosa cells of ovarian follicles, and the Leydig cells of testis (Young *et al*., 2005).

Gonadal steroidogenesis is a multistep process that involves the conversion of 27-carbon cholesterol to sex steroids catalyzed by several enzymes belonging to the cytochrome P450 superfamily and hydroxysteroid dehydrogenases (Payne and Hales, 2004). Besides gonads, the non-gonadal tissues like the adrenal cortex and interrenal tissues are considered as the major sites for the synthesis of steroid hormones (Norman and Litwack, 1997). The bioactive gonadal steroids include estrogens, androgens, progesterones, and other steroids that are derived from the parent cholesterol molecule. Estradiol, the most potent steroid hormone in females, controls the major reproductive processes namely sexual differentiation, maturation, and reproduction. In addition, it is also involved in numerous other

physiological processes like development, differentiation, and homeostasis of diverse target organs, especially in the regulation of neuronal growth and differentiation. However, in males, estrogens stimulate the proliferation of gonadal stem cells, which plays an important role in male fertility (Cheshenko *et al*., 2008). Estrogens are produced in the granulosa cells of the ovarian follicle under the influence of gonadotropins (Janz, 2000). In teleosts, the gonadal estrogens also stimulate the production of the yolk protein or vitellogenin in the hepatic tissues as a nutritional source for the developing embryo and promote intersex formation in some protandrous species (Kime, 1998).

The major androgens synthesized in the testis of teleost fish include testosterone, androstenedione, and 11-ketotestosterone under the influence of luteinizing hormone. Androgens are involved in spermatogenesis, maturation of male gamete, development of secondary sexual characters, and induction of reproductive behavior in male fish. Dehydroepiandrosterone (DHEA) is the precursor that is readily converted to androstenedione and then to testosterone in Leydig cells, which undergo aromatization to produce estradiol, the female hormone (Toor and Sikka, 2017).

In teleost fish, the major progesterone such as 17α,20β-dihydroxy-4-pregnen-3-one and 17α,20β,21-trihydroxy-4-pregnen-3-one are involved in oocyte maturation, ovulation, seminal fluid production, spermatozoa motility, spermiation, and spermatogonial proliferation (Fang *et al*., 2018). Steroids are readily released out from the gonadal cells after biosynthesis and bind to cytosolic or nuclear

membrane receptors exerting autocrine, paracrine, or endocrine regulation of hormonal action (Beato *et al*., 1996).

1.2 Steroidogenic pathway in gonads

The steroidogenic acute regulatory protein (StAR) is a key factor involved in gonadal steroidogenesis. StAR is a mitochondrial protein synthesized as a 37kDa precursor under the influence of luteinizing hormone (Rone *et al*., 2009). The steroidogenic pathway is initiated by the StAR protein, which transfer the cholesterol across the inner mitochondrial membrane, which is the rate-limiting step in steroidogenesis. In the inner mitochondrial membrane, the synthesis of steroid precursor pregnenolone from cholesterol occurs by cholesterol side-chain cleavage cytochrome P450 (P450scc) encoded by the *Cyp11a1* gene. Pregnenolone, the basic steroid, is converted into progesterone by the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme through the Δ4 pathway. Metabolism of pregnenolone also occurs by 17α-hydroxylase of cytochrome P450c17 (P450c17) that convert into 17α-hydroxypregnenolone through Δ5 pathway (Young *et al*., 2005).

Cytochrome P450c17 has dual enzymatic activity that catalyzes both 17α-hydroxylation and 17,20-lyase, which converts 21-carbon steroids to 19-carbon precursors of sex steroids namely dehydroepiandrosterone (DHEA) through Δ5 pathway or to androstenedione through Δ4 pathway. DHEA is converted by the catalytic activity of the 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme into androstenedione or androstenediol (Fan *et al*., 2014;

Tenugu *et al*., 2021). Testosterone is produced from androstenedione or androstenediol by the activities of 17β-HSD and 3β-HSD enzymes, respectively. The most common and very potent androgen found in male teleosts fish is the 11-ketotestosterone (11-KT), which requires the cytochrome P450 11β-hydroxylase (P45011β) enzyme for the conversion of testosterone into an intermediate 11β-hydroxytestosterone (Zhang *et al*., 2020).

The cytochrome P450-19 (CYP19) is the aromatase enzyme that catalyzes testosterone into estradiol (Miller and Auchus, 2011). Other important steroids such as progesterone, and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P), which is also known as maturation inducing steroid (MIS) are synthesized from androgens in males and estrogens in females (Ohta *et al*., 2002; Jeng *et al*., 2012). In the steroidogenic pathway during the pre-maturation phase, lyase (P450C17) activity regulates the shift from the production of androgens to progestogens, which is associated with LH surge. In male teleost, 20β-hydroxysteroid dehydrogenase (20β-HSD) is known to regulate the synthesis of 17α,20β-P (Vazirzadeh and Guiguen, 2017). Similarly, the mechanism of steroid shift is also found in females, where in the vitellogenic follicles, the conversion of 17-hydroxyprogesterone to MIS is regulated by the activity of the 20β-HSD enzyme (Sreenivasulu and Senthilkumaran, 2009).

1.2.1 Key steroidogenic enzymes

Gonads of teleost fishes possess more than fifteen steroidogenic enzymes in the steroidogenic pathway for the synthesis

of bioactive steroids. The four major oxidoreductases such as 3β-HSD, 17β-HSD, 20β-HSD, and 11β-HSD play crucial roles in the biosynthesis of sex steroids. The 3β-HSD or Δ5-Δ4 isomerase enzyme converts 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, and dehydroepiandrosterone to androstenedione. The enzyme 3β-HSD is regulated by gonadotropin in most teleosts, which is also involved in the hepatic degradation of the pheromone androstenone (Rasmussen *et al*., 2013). The 17β-HSD is the enzyme involved in the production of testosterone using androstenedione as the precursor molecule. Several isoforms of 17β-HSD are reported in teleosts namely 17β-HSD1, 17β-HSD2, and 17β-HSD3, which are involved in the interconversion of estrone, aromatization, and for the regulation of testosterone production, respectively (Zhou *et al*., 2005). The enzyme also regulates the level of specific substrates required for sex steroid biosynthesis through the interconversion of the inactive 17-keto-steroids to active 17β-hydroxy-steroids thereby involves in the process of sex differentiation and gametogenesis in fish (Adamski and Jakob, 2001).

The 20β-HSD enzyme is involved in the conversion of 17α-hydroxyprogesterone into 20β-hydroxysteroids or MIS. In teleosts, MIS initiates meiosis in the spermatocyte, milt production, sperm mobility, steroid catabolism, and in the generation of pheromones for intraspecies communication (Rajakumar and Senthilkumaran, 2013). The type-2 11β-HSD (11β-HSD2) is involved in the synthesis of 11-oxygenated androgens or 11-KT with a high level of expression during pre-spawning and spawning stages of the male fish whereas 11β-HSD1 is not characterized in teleosts (Fernandino *et al*., 2013).

Steroidogenic pathways also include a series of enzymes belonging to the cytochrome P450 superfamily namely the enzymes of cholesterol side-chain cleavage (P450scc/ CYP11A), cytochrome P450 c17a-hydroxylase, and 17,20-lyase (CYP17), and cytochrome P450 aromatase A-isoform (CYP19A) (Miller, 2017). CYP11A1 is the enzyme that initiates the steroidogenic pathway while CYP11B is the principal enzyme involved in androgenesis (Rajakumar and Senthilkumaran, 2015). In teleost fish, CYP17 consists of CYP17A1 and CYP17A2, primarily involved in the synthesis of androgen from progesterone, and for the steroidogenic shift from estradiol to testosterone (Rajakumar and Senthilkumaran, 2020). The CYP19A1 or aromatase enzyme is the most important and widely studied enzyme in many teleosts models as it produces C18 steroids, which play a major role in sexual development (Diotel *et al*., 2010).

1.3 Aromatase in fish reproduction

The cytochrome P450 aromatase (CYP19A1) is the most conserved enzyme common to all teleost species that converts testosterone or androstenedione into estradiol or estrone, respectively (Simpson *et al*., 2002). In teleost, aromatase is encoded by aromatase A or *cyp19a1a* and aromatase B or *cyp19a1b* genes, localized in the ovary and brain, respectively. The overlapping expression of aromatase mRNAs in the ovary and brain has been established in some teleost species (Cheshenko *et al*., 2008). Besides, other tissues such as the liver, kidney, digestive tract, adipose tissues, vascular tissues, bone, skin, and placenta express aromatase activity for genomic and non-

genomic estrogen signaling pathways, through which various cellular activities are regulated (Piferrer and Blazquez, 2005).

The upregulation of the *cyp19a1a* gene leads to ovarian differentiation whereas the downregulation of *the cyp19a1a* gene is essential for testicular differentiation, which is regulated at the transcription level (Wang *et al*., 2007). The brain aromatase activity of the *cyp19a1b* gene is associated with the circulating level of sex steroids, which regulates neuroplasticity, neurogenesis, sex differentiation, modulation of neuroendocrine function, and sexual behavior (Le Page *et al*., 2010). The expression of brain aromatase is more in the brain and pituitary of males than in females of teleosts, except in some species that shows parental care with high circulating estrogen that cross the blood-brain barrier, and increases aromatase transcription (Diotel *et al*., 2010; DeAngelis *et al*., 2018). The brain aromatase activity along with estrogen production influence the social behaviors in fish such as courtship, reproductive, aggressive, and parental care (Trainor *et al*., 2003; Ramallo *et al*., 2017).

In teleosts, the female reproductive cycle is largely regulated by the expression and activity of aromatase A or *Cyp19a1a* in the gonads, which is involved in vitellogenesis and regulation of sex steroids (Ijiri *et al*., 2003). In male teleosts, aromatase is expressed in the Leydig cells, Sertoli cells, and germ cells, which plays a crucial role in spermatogenesis, testicular steroidogenesis, and fertility (Caulier *et al*., 2015). Sexual dimorphism is also found associated with aromatase activity in the brain where the differences in aromatase expression lead to different mating and reproductive tactics, or

hierarchy placement, which indicate differential gene expression on sex (Partridge *et al*., 2016).

1.4 Regulation of aromatase gene expression

Teleost fishes have two aromatase genes, *Cyp19a1a* and *Cyp19a1b* that exist on the distinct chromosome resulting from ancestral whole-genome duplication (Chiang *et al*., 2001). Studies on the tissue distribution and gene expression have revealed that both genes are regulated by different transcription factors on 5´-flanking regions with similar enzyme coding exons. The promoter region of the *Cyp19a1b* has the sites for estrogen-responsive elements (ERE) at 300/350 bp from the transcription initiation site (Diotel *et al*., 2010). In addition, several other putative cis-regulatory elements such as androgen-responsive element (ARE), and dioxin or aryl hydrocarbon responsive elements (DRE/ AhRE) were located within 3000 bp proximal to the promoter sequence of *Cyp19a1b* gene demonstrating the sensitivity towards estrogen or xenoestrogen, and the role of estrogen receptors on the transcription of *Cyp19a1b* gene. The binding of estrogens to the estrogen receptors activates the receptor by dimerization and initiates transcription by binding to the ERE located on the *Cyp19a1b* promoter sequence, thereby activating the expression of the aromatase gene (Cheshenko *et al*., 2008).

The promoter region of *the Cyp19a1a* gene contains a steroidogenic factor-I (SF-1) regulatory element and lacks EREs (Diotel *et al*., 2010). The transcriptional factor SF-I is a tissue-specific orphan receptor located in the nucleus, which promotes the expression

of StAR protein and initiates *Cyp19a1a* gene transcription. In teleost gonads, the gonadotropin and steroidogenic factor are known to mediate the activation of *Cyp19a1a* gene expression (Yan *et al*., 2019). The level of *Cyp19a1a* gene expression varies in gonads, where high expression was found in the ovary than testis, particularly during vitellogenesis and spawning period. Similarly, the peak level of *Cyp19a1a* gene expression occurs during the maturation phase of testicular development. DNA methylation in the promoter region of the gonadal aromatase gene contributes to the maintenance of the sex ratio in fishes (Chen *et al*., 2018). Hypomethylation of the *Cyp19a1a* promoter develops the undifferentiated gonads into the ovary whereas hypermethylation prevents the transformation of an undifferentiated gonad into an ovary during male sex differentiation (Navarro-Martin *et al*., 2011).

1.5 Physiological role of gonadotropins

Reproduction in teleosts involves a cascade of hormones regulated through the hypothalamic-pituitary-gonadal (HPG) axis. Hypothalamus produces gonadotropin-releasing hormone (GnRH) that directly stimulates or inhibits the release of gonadotropins namely follicle stimulating hormone (FSH) and luteinizing hormone (LH). Gonadotropins are glycoproteins produced in gonadotropic cells of the adenohypophysis that activates the receptors in the gonads for the synthesis of sex steroids, growth factors, and effectors of gonadal development (Levavi-Sivan *et al*., 2010). FSH is a heterodimeric protein involved in early gonadal development, ova maturation, and

vitellogenesis in females. Despite this, FSH acts on the Sertoli cells of the testis to promote sperm production and maturation of spermatozoa (Park *et al*., 2005).

In the ovary, LH acts on the theca cells for the synthesis of steroid hormones, stimulates ovulation, and modulates estrogen synthesis through aromatization. In males, LH acts on the Leydig cells of the testis to initiate spermatogenesis and stimulates the synthesis and secretion of testosterone (Schulz *et al*., 2001). Besides gametogenesis and gonadal steroidogenesis, gonadotropins are also involved in ovulation, spermiation, and spawning processes in teleost fishes. The physiological functions of gonadotropins on teleost reproduction are also influenced by several other factors including the species, sex, reproductive status, and the complex endocrine interactions along the HPG axis (Zohar *et al*., 2010).

The synthesis and release of GnRH are influenced by a neuropeptide namely kisspeptin through the HPG axis, and found abundant in the brain, particularly in the hypothalamus (Ogawa *et al*., 2012). However, the other neurohormone such as gonadotropin inhibitory hormone (GnIH) expressed mainly in the brain and pituitary is known to inhibit the release of gonadotropin by negatively regulating the release of GnRH and kisspeptin (Ubuka *et al*., 2016). Thus both GnIH and GnRH along with kisspeptin are the essential components that regulate the reproduction of the teleost fishes.

1.6 Effects of xenoestrogen on the reproduction of fish

Xenoestrogens are estrogen analogs or estrogen-like substances that mimic the natural estrogens, the primary sex hormone of females. Estrogen-regulated processes are very important for many physiological functions associated with female reproduction including reproductive behavior, gamete development, ova maturation, sexual differentiation, steroidogenesis, and other endocrine functions. However, xenoestrogens are chemical substances that mimic structural similarity and properties of the natural estrogens. They may be either natural or synthetic products like pesticides, pharmaceuticals and personal care products, plasticizers, etc. The xenoestrogens are known to enter into the aquatic environment through wastewater disposal, rainwater run-off, transportation, accidental spillage, and groundwater discharge thereby affects the life of aquatic organisms, including fish (Petersen *et al*., 2017; Shincy and Chitra, 2020).

Xenoestrogens can undergo bioaccumulation in fish tissues thereby elicit various reproductive abnormalities in both sexes of fish. There are several routes by which xenoestrogens enter into fish, which mainly includes dermal contact, gills, and oral ingestion. Exposure to xenoestrogens in male fish abnormally increases the production of vitellogenins, and declines sperm count, whereas in females reduction in the rate of fecundity and hatchability of eggs occurs (Paschoalini *et al*., 2021). The gonochoristic teleosts that inhabit chemically contaminated ecosystems are suspected with an alarming increase in the detection of ovotestis, testisova, intersex, and testicular oocyte conditions. Intersexuality is a common phenomenon among teleost fish

exposed to xenoestrogens as evident by feminization, i.e., development of oocytes within testicular tissue, or masculinization i.e., development of spermatogenic cells within the ovarian tissue (Grilo and Rosa, 2017; Hicks *et al*., 2017). The formation of intersexuality also results in other reproductive abnormalities including delayed spermatogenesis and oogenesis in males and females, respectively (Bahamonde *et al*., 2015).

Xenoestrogens are the endocrine disruptors having a very long half-life in the environment, and due to their lipophilic properties, they tend to bioaccumulate and biomagnify in the aquatic food web. Further concerns are associated with their metabolites, where most of them are more potent than their parent compounds. Exposure of xenoestrogens or their metabolites changes the strategy of the fish life cycle where the failure of metabolic detoxification process under the toxic condition affects the growth, survival, and adaptation. Thus chronic exposure to xenoestrogens even at low concentration could affect fish reproduction that ultimately results in the decline of the fish population. Some studies have suggested that the improved wastewater treatment technologies can control the potent effects of xenoestrogens to some extent (Schwindt *et al*., 2014; Hamilton *et al*., 2016).

1.7 Triclosan as a xenoestrogen

Triclosan is a xenoestrogen, which mimics some of the major persistent endocrine-disrupting chemicals such as polychlorinated biphenyls, bisphenol A, and dioxins, and also shows structural similarity with the endogenous thyroid hormone (Crofton *et al*., 2007). However, triclosan has both estrogenic as well as anti-androgenic

properties (Ishibashi *et al*., 2004; Wang *et al*., 2018). The presence of triclosan has been detected in soils and sediments in the range of 2.7 to 26.8 µg L^{-1} (Dhillon *et al*., 2015), in groundwater and surface water between 0.055–0.184 and 0.041–0.077 µg L^{-1}, respectively (Sarkar *et al*., 2020). The ubiquitous presence of triclosan in the aquatic bodies and its endocrine disrupting properties target the hypothalamic-pituitary-gonadal axis thereby impairs fish reproduction (Wang *et al*., 2018; Song *et al*., 2020).

The estrogenic activity of triclosan studied in some fish species found an increase in the level of hepatic vitellogenin in male fish and also affects female fish in the embryonic development and hatchability of fertilized eggs (Ishibashi *et al*., 2004). Xenoestrogen-induced vitellogenin production in male fish is associated with other reproductive defects including skewed sex ratio, intersex, reduction in the quantity of milt, and sperm density, associated with other gonadal and renal pathologies (Wheeler *et al*., 2005). Methyl triclosan, the metabolite of triclosan also accumulates in the fish with concentrations ranging from 30 to 600 ng g^{-1}, which is highly toxic causing reproductive and developmental defects (Balmer *et al*., 2004; Leiker *et al*., 2009). Triclosan exposure has been shown to decrease nest defense behavior thereby affects brood survival in the fathead minnow, *Pimephales promelas* (Schultz *et al*., 2012).

1.8 Relevance of the study

Globally, freshwater ecosystems are adversely polluted by several environmental contaminants. Triclosan, one of the

environmental pollutants classified as endocrine disruptors is known to interfere with the functions of endogenous reproductive hormones. Most of the xenoestrogens are lipophilic and tend to bioaccumulate and biomagnify in the food web. The adverse effects of the chemical pollutants are studied using fish as the sentinel species. The present study was hypothesized to evaluate the chronic effects of triclosan on the reproductive functions of the fish, *Anabas testudineus* during the pre-spawning period. The findings share critical information about variations in some reproductive parameters including aromatase gene expression in the brain and gonads, levels of gonadotropins and sex hormones in blood serum as well as gonadal histopathology of the fish, which are suspected to affect the fish population. The broad range of biochemical parameters examined in the pre-spawning stage of the fish also aims to provide better insights at protecting the aquatic organisms against xenoestrogenic chemical compounds to maintain a well-balanced population in the aquatic ecosystems.

2. OBJECTIVE OF THE STUDY

- To evaluate the effect of chronic exposure of triclosan on gonadal development and steroidogenesis in the pre-spawning phase of the fish, *Anabas testudineus*

3. REVIEW OF LITERATURE

Triclosan, an antimicrobial agent extensively used in several consumer products has been shown to cause adverse effects on growth and reproduction in aquatic organisms including microorganisms, algae, daphnids, and fish (Orvos *et al*., 2002). Several studies have also reported the adverse effects of triclosan on the reproductive physiology of fish and mammals mediated through the hypothalamus-pituitary-gonadal (HPG) axis (Kumar *et al*., 2009; Stoker *et al*., 2010). In some fish species, triclosan exposure caused a reduction in spawning rate and egg hatchability causing developmental defects, declined sperm count, and infertility (Wang *et al*., 2018). Besides, the ability of triclosan to interfere with the transcriptional activity of estrogen receptors has been documented in several fish species.

In a study, developmental exposure of triclosan to medaka fry for 14 d has suggested anti-estrogenic or androgenic effects based on fin trait and secondary sexual characters (Foran *et al*., 2000). Triclosan has been shown to disrupt steroid hormone action *in vitro* and *in vivo*, which suggested estrogen-antagonistic activity (Matsumura *et al*., 2005). Chronic exposure of seven pharmaceuticals and personal care products including triclosan at environmentally relevant concentrations to fathead minnows collected from the Canadian municipal wastewater effluents has not affected the survival, growth, or egg production, however, deformities in the F1 generation of the fish has been reported (Parrott and Bennie, 2009). A study has reported that aquatic species such as algae, invertebrates, and fish species

showed more sensitivity to triclosan than mammals, where it exerted high toxicity to algae, and caused reproductive and developmental defects in some fish species (Dann and Hontela, 2011).

The adult fathead minnows *Pimephales promelas* exposed to triclosan for 48 h has been shown to downregulate the expression of androgen receptor and steroidogenic acute regulatory protein in testes stating estrogenic effects of the compound (Zenobio *et al*., 2014). Wild male Largemouth Bass, *Micropterus salmoides* collected from Las Vegas Bay, Unites States contaminated with 252 organic chemicals including triclosan caused defects in the endocrine and reproductive system by 50% reduction in the sperm count thereby suggested disruption in male post-gonadal growth and reproductive functions (Goodbred *et al*., 2015).

Endocrine-disrupting effects of triclosan has been found to be mediated through estrogen- as well as non-estrogen receptor pathways in the female Yellow River carp, *Cyprinus carpio* under semi-static condition for 42 d. The study revealed that triclosan interfere the HPG axis at multiple potential loci through three possible mechanisms such as by enhancement of the mRNA expression of the hypothalamus and gonadal aromatase that subsequently increased the serum concentrations of estradiol for vitellogenin production in hepatopancreas. The second mechanism has occurred through the induction in the expression and secretion of gonadotropin-releasing hormone thereby disturbed the reproductive endocrine axis for the increase in estradiol secretion. Besides, triclosan has been shown to enhance the synthesis and binding to the estrogen receptor, and the

complex formed activated the gene involved in the synthesis of vitellogenin in hepatopancreas (Wang *et al*., 2017). The adverse effects of triclosan on reproduction and endocrine system have been found associated to altered expression of four miRNAs namely *miR-125b*, *miR-205*, *miR-142a*, and *miR-203a* that were mainly involved in fatty acid synthesis and metabolism (Lin *et al*., 2017). Zebrafish exposed to triclosan for 7 d in the early life stages has been shown to delay in the hatching rate, and induced neurotoxicity in the developing embryo (Falisse *et al*., 2017).

Triclosan exposed to female Yellow River carp, *Cyprinus carpio* enhanced vitellogenin synthesis by interference with hypothalamic-pituitary-gonadal axis as well as by binding with estrogen receptor, which indicated that the estrogenic effects are mediated through ER and non-ER pathways (Wang *et al*., 2017). The interaction of triclosan with the estrogen receptor signaling pathway illustrated using *in vitro* and *in vivo* zebrafish-specific reporter gene assays showed lack of agonistic and antagonistic activities towards zfER α, β1, and β2 subtypes by increasing vitellogenin level and gonadal aromatase through non-ER pathway (Serra *et al*., 2018). Similarly, another study has reported that triclosan exposed to male Yellow River carp *Cyprinus carpio* under the semi-static condition for 42 d induced vitellogenin production by non-ER-mediated pathway as evidenced by increased expression of the hypothalamus and gonadal aromatase, which consequently increased serum estradiol to induce vitellogenin production in hepatopancreas. Besides, the altered expression and secretions of gonadotropins, and reduction in the

aromatase mRNA levels indicated potential aromatase-mediated antiandrogen action in the male carp (Wang *et al*., 2018). In a study, transgenic zebrafish models have been used to demonstrate the mechanism of developmental neurotoxicity induced by triclosan, which showed up-regulation in the expression of neurodevelopmental genes stating neurotoxic effects in the developing embryos (Kim *et al*., 2018).

In the freshwater ecosystem near the Pearl River catchment, China, some of the endocrine-disrupting contaminants such as triclosan, triclocarban, parabens, and bisphenol A have been found to increase the body size-dependent bioaccumulation, tissue distribution, trophic magnification, and maternal transfer in the freshwater fishes, which varied based on species, targeted tissues, and nature of toxicant. Among the endocrine disruptors, triclosan has shown potential biomagnification with the trophic magnification factor of 3, 4.3, and 4 in the liver, belly fat, and dorsal muscle, respectively (Peng *et al*., 2018).

Triclosan exposure to four food fishes namely *Cyprinus carpio*, *Ctenopharyngodon idella*, *Labeo rohita*, and *Cirrhinus mrigala* caused teratogenicity in the early life stages as the exposed hatchlings were observed to be very weak and paralyzed without movement, and remained settled at the bottom of tanks, along with abnormal and delayed embryonic development, delay and decline in the hatchability of the eggs (Dar *et al*., 2019). Methyl triclosan has been shown to affect the swimming pattern in zebrafish embryos due to spine malformation or tail circulation (Fu *et al*., 2019). Exposure to eight

priority pharmaceutical drugs including triclosan at environmentally relevant concentrations in zebrafish larva altered normal behavioral patterns as revealed by the reduction in the swimming speed, increased escape behavior, and erratic movement (Zhou *et al*., 2019).

Larval exposure to environmentally relevant concentrations of triclosan has showed impaired metamorphosis in zebrafish associated with reduced swimming speed and reproductive performance including a shift in the sex ratio, reduced egg production, impaired oogenesis and spermatogenesis, altered vitellogenesis, gonadal development, gamete quality, sperm production, sperm motility, and reproductive behaviors by disrupting hypothalamus-pituitary-gonadal axis (Stenzel *et al*., 2019). *Clarias gariepinus* exposed to triclosan at sublethal and environmentally relevant concentrations have been shown to cause embryotoxic effects as evidenced by a decrease in the hatching success and reduction in the number of heart beats per minute and severe abnormalities in the developing embryos (Jimoh and Sogbanmu, 2021).

Based on the existing literature, it was clear that triclosan caused developmental and reproductive effects in various fish species. The mode of action of triclosan varied among the species by exerting both estrogenic as well as anti-androgenic effects, which has been influenced by several factors including the concentration of the toxicant, species, duration of exposure, receptor specificity, and single or combined effects of the compound. The present chapter provides suitable evidence to prove the mode of action of triclosan in the freshwater fish, *Anabas testudineus* exposed chronically during the pre-spawning period of reproduction.

4. MATERIALS AND METHODS

4.1 Test animal

Fingerlings of *Anabas testudineus* (2 ± 0.6 cm length; approximately 2 months old) were collected from the Pulimugham Aquafarms and hatcheries, Alappuzha district, Kerala, India. Fingerlings were reared in the laboratory until they attained maturity, provided with commercial fish pellets twice daily. The fish stock was maintained in a well-aerated and dechlorinated tap water under the natural photoperiod (12 h dark: 12 h light) in glass tanks (40 L; 30 cm width × 60 cm length × 30 cm depth). According to the guidelines of water quality testing (APHA, 1998), the water temperature (28 ± 3 °C), pH (7.4 to 7.6), dissolved oxygen (8.64 ± 0.6 mg L^{-1}), and salinity (<100 ppm) were maintained throughout the study. The care and use of the fish complied with the Animal Welfare Board of India and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the Ministry of Environment, Forest, and Climate change, Government of India.

4.2 Chemicals

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol; 97% purity) and dimethylsulfoxide (DMSO, ≥99%) were purchased from HiMedia Research Laboratories Pvt. Ltd, Mumbai, India. Hormone assay kits and aromatase assay kits were procured from Bioassay Technology Laboratory, Shanghai, China. TRIzol® reagent

(Invitrogen, Thermo Fisher Scientific, USA), 1-bromo-3-chloropropane, and 2-propanol were purchased from Sigma-Aldrich Pvt. Ltd. TB Green Premix Ex Taq II (Tli RNase H Plus), diethylpyrocarbonate (DEPC) treated water, ribonuclease (RNase) inhibitor, RNase-free-DNase, RevertAid Moloney murine leukemia virus (M-MuLV) reverse transcriptase, Oligo(dT) primer, nuclease-free water, deoxy-NTPs, and TaqDNA polymerase were procured from DSS Takara Bio India Pvt. Ltd. Invitrogen RNAlater stabilization solution and RevertAid First Strand cDNA synthesis kit were obtained from Thermo Fisher Scientific, USA. All the other chemicals were purchased from local commercial sources.

4.3 Design of experiments and grouping of fish

A. testudineus of pre-spawning phase i.e., from April to June (8 ± 1 g and 8.5 ± 0.75 cm) of approximately 10-12 months old were used in the present study (Pal *et al*., 2018). Fish were randomly collected from the stock and grouped in separate glass tanks, each group holding ten fish. Triclosan at three different concentrations was exposed to the fish for 90 d along with control groups, which include toxicant-free (only tap water) and solvent-control (DMSO; 0.001% v/ v) group. The selection of test concentrations was based on previous literature, in which two environmentally relevant concentrations (0.009 and 9 µg L^{-1}) that represent the lowest dose detected in the surface waters of Indian rivers (Ramaswamy *et al*., 2011; Nag *et al*., 2018), and one-tenth of LC_{50}-96 h, i.e., 176.7 µg L^{-1} representing the sublethal concentration (Priyatha and Chitra, 2018) were used in the study. The data

represented in Figures denotes the mean values of 10 animals per group, in replicates.

The experiments were performed in a static renewal system holding 40 L of water in a glass tank. The water was renewed at every 96 h from both control and treatment groups by siphoning 80-90% of water and refilled with the same volume of water and test concentrations in the respective tanks. The particulate wastes deposited at the bottom of the tank were removed carefully without giving stress and disturbance to fish. Sex differentiation was not prominent during the pre-spawning period so male and female fish were distinguished and separated based on the large belly size of females, and others were grouped as males. The experimental design and grouping were detailed in the following table:

Group	**Fish of pre-spawning stage n = 10/ group, in replicates, maintained for 90 d**	
Control	No toxicant (only tap water)	
Vehicle	Solvent - dimethylsulfoxide (DMSO 0.001 % v/ v)	
Treatment (Triclosan)	0.009 µg L^{-1}	Environmentally relevant concentration (Ramaswamy *et al*., 2011)
	9 µg L^{-1}	Environmentally relevant concentration (Nag *et al*., 2018)
	176.7µg L^{-1}	Sublethal concentration (Priyatha and Chitra, 2018)

4.4 Processing of samples

At the end of the exposure period i.e., after 90 d, fish from the respective control and treatment groups were weighed, with and without mucous deposition. Blood samples were collected from caudal vein using non-heparinized vials for the preparation of serum for hormone analysis. Fish were then killed by severing the spinal cord to collect gonads and brains, which were weighed and recorded. The sex of the fish was confirmed following the dissection of gonads, and both brain and gonadal tissues were removed and stored at −80 °C in suitable buffers until further processing.

4.5 Body weight and mucus deposition

After the treatment period, the body weight and mucus deposition was recorded as described by Al-Rasheed *et al* (2018), which was explained in chapter 3.

4.6 Collection of blood serum

Blood samples were collected from both male and female fish of the control and treatment groups. Fish were anesthetized with a minimum dosage of tricaine methanesulfonate (MS-222) at 500 mg L^{-1} buffered with 1M sodium hydroxide; pH 7-8, for the collection of blood from the caudal vein using non-heparinized vials. The collected blood was kept undisturbed at room temperature for 20-30 min and centrifuged at 2000-2500 rpm for 15 min to obtain serum samples. The collected serum samples were then transferred into micro vials and stored at −80 °C until hormone analyses were performed.

4.7 Collection of tissue samples

Gonadal tissues and brain were dissected out from five male and five female fish of each replicate from control and treatment groups, cleared in normal saline, blotted on a tissue paper, and weighed.

The relative weight of gonads (testes and ovary) or gonadosomatic index (GSI) was evaluated using the standard formula and expressed in percentage (King 1995; Sulistyo *et al*., 2000).

$$GSI = (\text{Tissue weight (g)} / \text{Fish weight (g)}) \times 100.$$

4.8 Processing of tissue samples

Gonadal tissues were cut into three portions, a portion of gonads and brain tissue were homogenized separately, centrifuged at 800*g* for 15 min at 4 °C, and the supernatants obtained were stored at −80 °C until biochemical analysis such as the activities of aromatase and steroidogenic enzymes, and total protein was performed. The second portion of the gonadal tissues was preserved in 10% buffered formalin for histological analysis. The third portion of gonadal tissues along with brain tissues of both male and female fish were immediately stored separately in RNAlater at −80 °C for the analysis of aromatase gene expression using qPCR.

4.9 Determination of serum hormones

Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and estradiol were determined by fish FSH-ELISA kits (Catalogue Number: E0039FI, Bioassay Technology

Laboratory, China), fish LH-ELISA kits (Catalogue Number: E0040FI, Bioassay Technology Laboratory, China), fish testosterone ELISA kits (Catalogue Number: E0001FI, Bioassay Technology Laboratory, China), and fish estradiol ELISA kits (Catalogue Number: E0050FI, Bioassay Technology Laboratory, China), respectively, strictly according to the manufacturer's instructions.

4.10 Biochemical analysis

4.10.1 Aromatase enzyme in brain and gonads

The concentration of fish aromatase enzyme in the brain and gonads was assayed using fish aromatase ELISA kits (Catalogue Number: E0034FI, Bioassay Technology Laboratory, China), following the manufacturer's instructions. The aromatase standards and samples were added to the wells of ELISA plates pre-coated with fish aromatase antibodies. Followed by the addition of biotin conjugate and enzyme conjugate reagents, the plate was incubated at 37 °C for 60 min. After incubation, the plates were rinsed 5 times with wash solution, then chromogenic reagent was added and the absorbance was measured at 450 nm within 15 min. Total protein was estimated using bovine serum albumin as the standard (Lowry *et al*., 1951).

4.10.2 Gonadal steroidogenic enzymes

The activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were measured in the gonads according to Bergmeyer (1974) with minor modifications. Briefly, the gonadal homogenate (10%) was prepared in 5 mM

potassium phosphate buffer containing 1 mM EDTA and 20% spectroscopic grade glycerol and supernatant was collected after centrifugation at 10,000*g* for 10 min at 4 ºC. For the assay of 3β-HSD, an aliquot of the supernatant was mixed with pyrophosphate buffer (100 mM), NAD (0.5 mM), and dehydroisoandrosterone (0.1 mM), and absorbance was read immediately at 340 nm for 5 min at 30 s interval in a spectrophotometer against the blank. The units are expressed in μmol of NAD reduced/ min/ mg protein. The assay mixture of 17β-HSD contained pyrophosphate buffer (100 mM), NADPH (0.5 mM), and 1,4-androstenedine-3,17-dione (0.8 mM) was read at 340 nm immediately after the addition of sample at 30 s interval for 5 min in a spectrophotometer against the blank. The enzyme activity was expressed as μmol of NADP formed/ min/ mg protein.

4.11 Histology of gonads

Ovary and testis of each exposure group were fixed in 10% buffered formalin for 24-48 h, which were later used for histological analysis. The tissues were dehydrated in ascending grades of alcohol and cleared in xylene until they became translucent. Then the gonadal tissues were embedded in molten paraffin wax for an hour for the complete impregnation to make the tissue blocks. Tissue sections of 4 to 6 μm thickness were made using a rotary microtome, double-stained with hematoxylin and eosin, and finally mounted using DPX (Roberts and Smail, 2001). Five slides of gonadal tissues were prepared from each group and examined under a light microscope equipped with a

digital camera (Magcam DC series camera). Histological fields showing lesions were focused by random selection of frames that varied between 5-6 frames and photographed at 40-100 X magnification for ovary, and 100-400 X magnification for testes. Histological lesions of the ovary and testis were confirmed as per the criteria as previously described in the OECD guidelines (OECD, 2009), and the semi-quantitative scoring of lesions were categorized as nil/ absent (-), mild (+), moderate (++), and severe (+++) damages (Leon *et al*., 2007).

4.12 Aromatase (*cyp19a1a* and *cyp19a1b*) gene expression

Total RNA was extracted manually from each sample of approximately 50 mg tissue using TRIzol reagent. Briefly, tissue was homogenized and incubated with TRIzol reagent at room temperature for 10 min. Chloroform was added to the mixture, and centrifuged at 12,000 rpm for 15 min at 4 ºC. The supernatant collected was incubated with ice cold isopropanol for 10 min at 4 ºC followed by centrifugation at 12,000 rpm for 10 min at 4 ºC. The pellets were collected in 75% ethanol, air dried and dissolved in RNAase-free water (30-50 µl), later stored at –20 ºC for further procedures. The quality and concentration of total RNA extracted was estimated by the NanoDrop one Microvolume UV-Visible Spectrophotometer (Thermo Fisher Scientific).

The first-strand cDNA was synthesized from the isolated RNA using Thermo Scientific RevertAid First Strand cDNA synthesis Kit according to the manufacturer's instructions. RNA (0.1 ng - 5 µg) was

reverse transcribed to cDNA in a final reaction volume of 20 µl. The samples were then incubated at 42 °C for 60 min and terminated the reaction by heating at 70 °C for 5 min. The obtained cDNA samples were used for the qPCR analysis using the housekeeping gene *gapdh* as the internal control. The selection of specific primers for the amplification of aromatase (*cyp19a1a* and *cyp19a1b*) and *gapdh* genes were based on the previous report as shown in the Table below (Pal *et al*., 2018).

List of primers for *cyp19a1a* and *cyp19a1b* transcripts

Gene		Primer Sequence (5′-3′)	Reference
cyp19a1a	Forward	TGACAGATGCTCAGGGACAG	Pal *et al.*, 2018
	Reverse	CTGTGAAGCCAGTCCAGTCT	
cyp19a1b	Forward	TGCTCTGGAGGAAAGTGAGG	
	Reverse	TCAGCAGATTGAGAGCGTCC	
gapdh	Forward	AGGGGCTCAGTATGTTGTGG	
	Reverse	AGGAGGCATTGCTGACAACT	

qPCR was performed using the SYBR Green PCR master mix (Takara Bio INC), and the reaction was carried out by using the QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA). For each qPCR reaction, 1 µl of cDNA (~ 50 ng) was mixed with 12.5 µl 2X SYBR Green PCR master mix (Takara Bio INC) and 5 pmol each of forward and reverse primers in a final volume of 25 µl. The reactions were amplified for 2 min at 50 °C, 10 min at 95 °C, 0.30 min at 95 °C, 0.45 min at 50 °C for 40 cycles. Amplification was followed

by thermal denaturation for 0.15 min at 95 °C, 0.15 min at 50 °C, and 0.15 min at 95 °C, and the melting curves generated were used to verify the amplification specificity. The housekeeping gene *gapdh* was used as the reference gene. The gene expression of aromatase relative to *gapdh* gene was estimated based on the comparative C_T method ($\Delta\Delta C_T$). The relative gene expression was measured based on the equation log2RQ, where RQ was calculated as $2^{-\Delta\Delta C}{}_T$, and $\Delta\Delta C_T = (C_{T,targetRNA} - C_{T,referenceRNA}) - (C_{T,calibrator} - C_{T,referenceRNA})$ (Winer *et al*., 1999; Livak and Schmittgen, 2001).

4.13 Statistical analyses

Data analyses were performed using SPSS v21.0 software. The values were expressed as mean ± standard deviation (SD), and significance among the control and the treatment groups were set using one-way ANOVA followed by Duncan's Multiple Range post-hoc test. Normal distribution and homogeneity of variance were ensured before conducting the analysis, and the values were considered as significant at $P < 0.05$, which was denoted as asterisks (*) in the Figures. All biochemical analyses were done in triplicates to avoid statistical errors.

5. RESULTS

5.1 Effect of triclosan on the body weight, mucus deposition and organ weights of the fish *Anabas testudineus*

The body weight of both male and female fish after 90 d of triclosan exposure at 0.009, 9 and 176.7 µg L^{-1} concentrations showed significant ($P<0.05$) reduction in a concentration-dependent manner when compared with the corresponding control groups (Fig. 1). However, the percentage of mucus deposition in the body of fish increased significantly ($P<0.05$) with an increase in the concentration of triclosan exposure (Fig. 2). The absolute and relative weights of gonads, and the weight of brain tissues showed significant ($P<0.05$) reduction in all treatment groups in both sex (Figs. 3-5).

5.2 Effect of triclosan on the activities of gonadal steroidogenic enzymes

The activities of gonadal steroidogenic enzymes, namely 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were decreased significantly ($P<0.05$) in the ovary and testis after 90 d of all tested concentrations, which was concentration-dependent on comparison with the respective control groups (Fig. 6).

5.3 Effect of triclosan on the concentration of aromatase enzyme in brain and gonads

The concentration of the aromatase enzyme exhibited a significant ($P < 0.05$) increase in the brain of female fish after 90 d of triclosan exposure at all concentrations whereas significant ($P<0.05$) reduction was observed in the ovary (Fig. 7). In the male fish, the activity of aromatase enzyme increased significantly ($P<0.05$) in brain and testis at all concentrations after triclosan exposure (Fig. 7).

5.4 Effect of triclosan on the expression of aromatase (*cyp19a1a and cyp19a1b*) genes in brain and gonads

The expression of aromatase genes namely *cyp19a1a* and *cyp19a1b* were up-regulated in the brain of female fish but reduced in the ovary (Figs. 8 and 9). The *cyp19a1b* gene expression was significantly ($P<0.05$) up-regulated in testis and brain of male fish in all treatment groups while no significant changes were observed in *cyp19a1a* gene expression when compared to the corresponding control groups (Figs. 8 and 9).

5.5 Effect of triclosan on the levels of serum hormones

The levels of serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) showed significant ($P<0.05$) reduction in both male and female fish in a concentration-dependent manner than the corresponding control groups (Fig. 10). The level of estradiol in the serum of female fish declined significantly ($P<0.05$) whereas male fish showed a significant ($P<0.05$) and concentration-dependent increase

(Fig. 11). However, a significant ($P<0.05$) reduction in the level of testosterone was observed after triclosan exposure in the serum of both male and female fish when compared to the respective control groups (Fig. 11).

5.6 Effect of triclosan on the histology of ovary and testis

Exposure of triclosan at 0.009, 9 and 176.7 µg L^{-1} concentrations for 90 d showed remarkable histological lesions when compared with the control tissues in the pre-spawning gonadal stage of *A. testudineus* (Tables 1 and 2). Histomorphology of ovarian tissue obtained from control groups, including negative-control and solvent-control, showed vitellogenic oocytes within well-developed, compact ovarian lamellae (Figs. 12A and 12B). Fish exposed to 0.009 µg L^{-1} concentration of triclosan were observed with loosely arranged oocytes (Fig. 12C). The membrane of oocytes showed prominent blebbing as a sign of regression of oocytes along with the development of loosely arranged oocytes after 9 µg L^{-1} concentration of triclosan treatment (Fig. 12D). In the sublethal exposure group i.e., 176.7 µg L^{-1} concentration of triclosan, there showed no vitellogenic oocytes but the follicles were formed of the developing chromatin nucleolus and perinucleolus stages along with the aggregation of melanomacrophage centres (Fig. 12E; Table 2). The major pathologies of the pre-spawning oocytes observed after 90 d of triclosan exposure in the fish such as loosely arranged oocytes, empty follicles, RBC infiltration, and melanomacrophage aggregation were represented separately in Figs. 13A-13D.

The histological examinations conducted in the testis after triclosan exposure also revealed severe abnormalities in the fish, *A. testudineus* (Table 2). The control testis of pre-spawning phase were normal showing compact seminiferous tubules with orderly arranged different spermatogenic stages (Figs. 12a and 12b). The changes observed in testicular morphology after 0.009 µg L^{-1} concentration of triclosan were the development of vacuolization in the interstitial tissue and within the seminiferous tubules (Fig. 12c). On exposure to 9 µg L^{-1} concentration of triclosan for 90 d developed thickening in the wall of seminiferous tubules along with vacuolization and loss of spermatozoa (Fig. 12d). The regression of seminiferous tubules and thickening of the interstitium with massive destruction of germ cell epithelium and loss of spermatozoa were identified after 176.7 µg L^{-1} concentration of triclosan exposure (Fig. 12e). Some of the significant morphological aberrations such as loss of spermatozoa, vacuolization, dedifferentiated seminiferous tubules by the formation of thickened interstitium and regression of the tubules, and development of melanomacrophage aggregation was shown separately in Figs. 13a-13d.

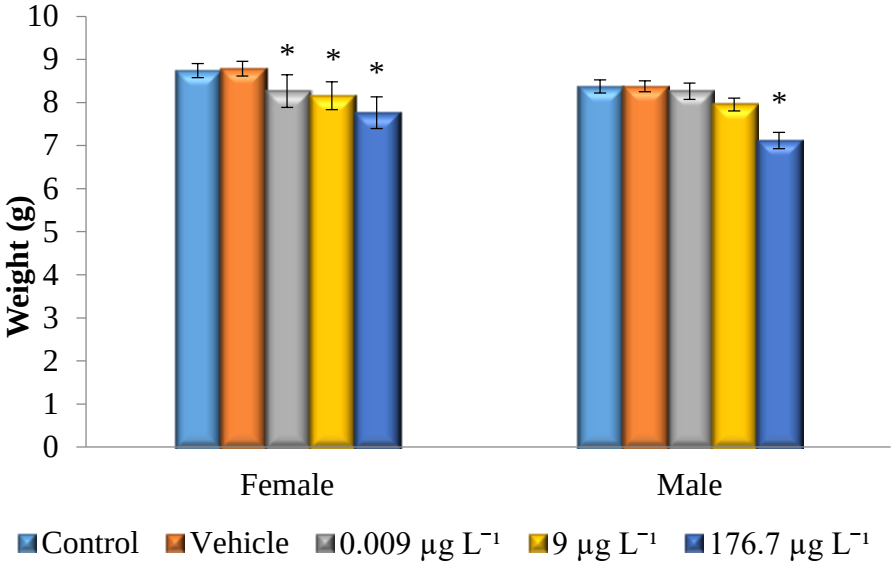

Fig. 1 Effect of triclosan on the body weight of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)

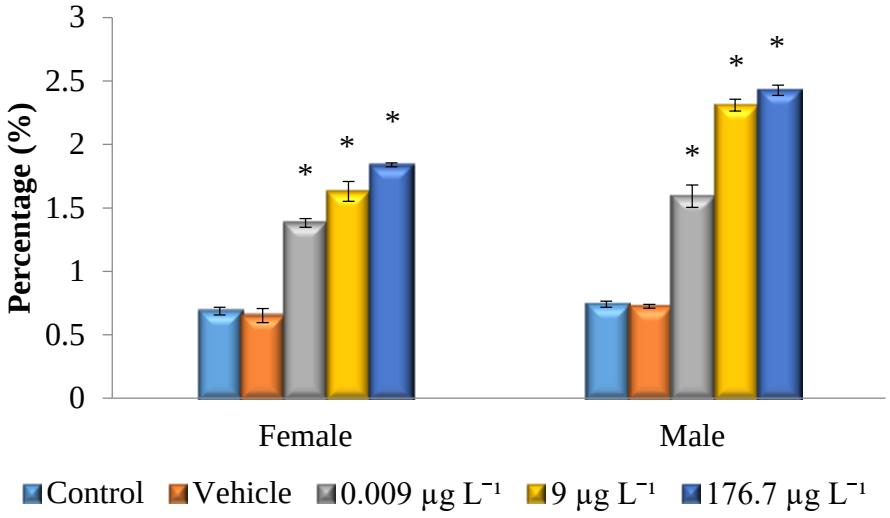

Fig. 2 Effect of triclosan on the mucus deposition in the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)

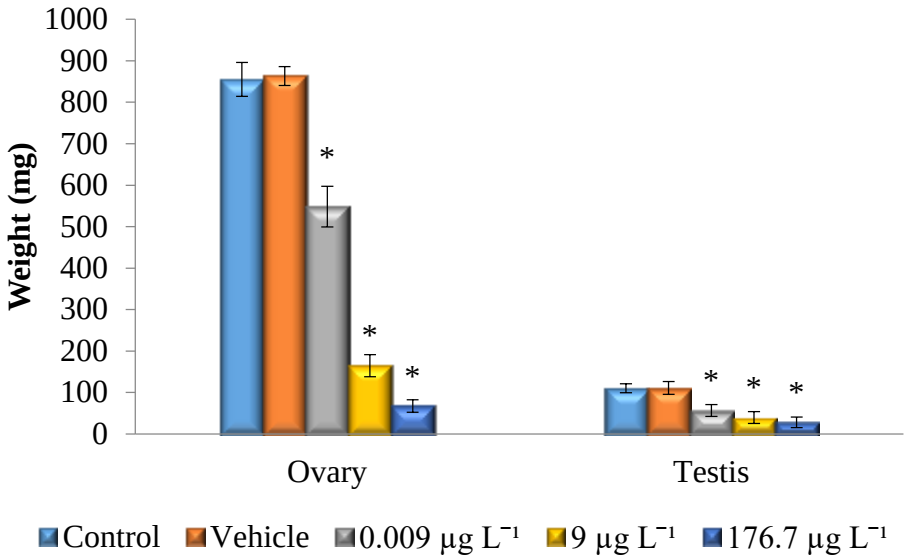

Fig. 3 Effect of triclosan on the weights of gonads in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)

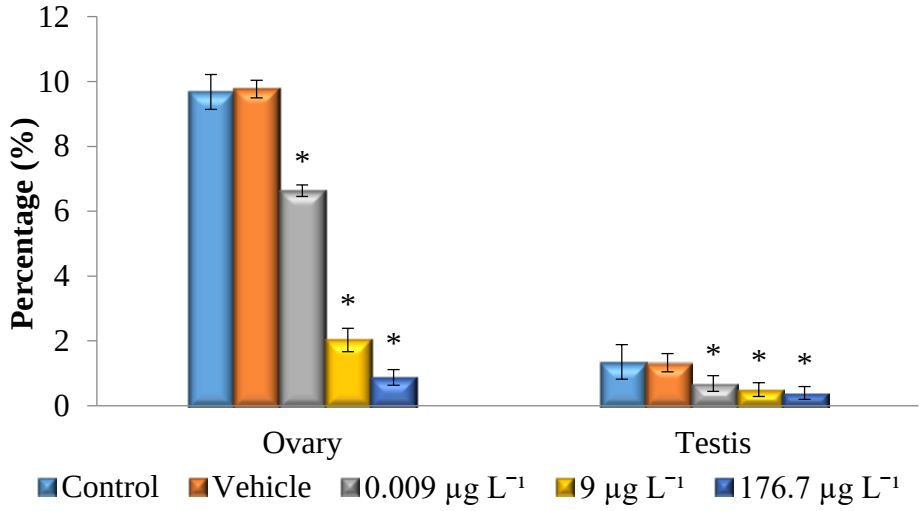

Fig. 4 Effect of triclosan on the gonadosomatic index (GSI) of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)

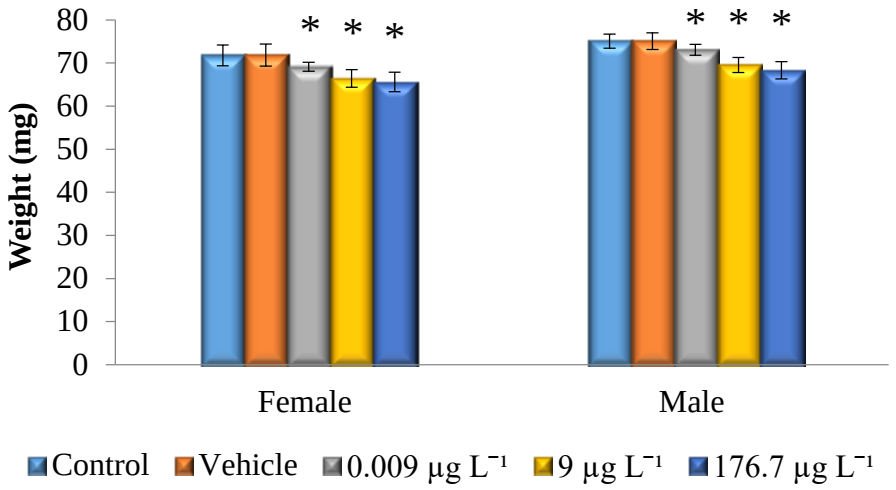

Fig. 5 **Effect of triclosan on the weight of brain tissue in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)**

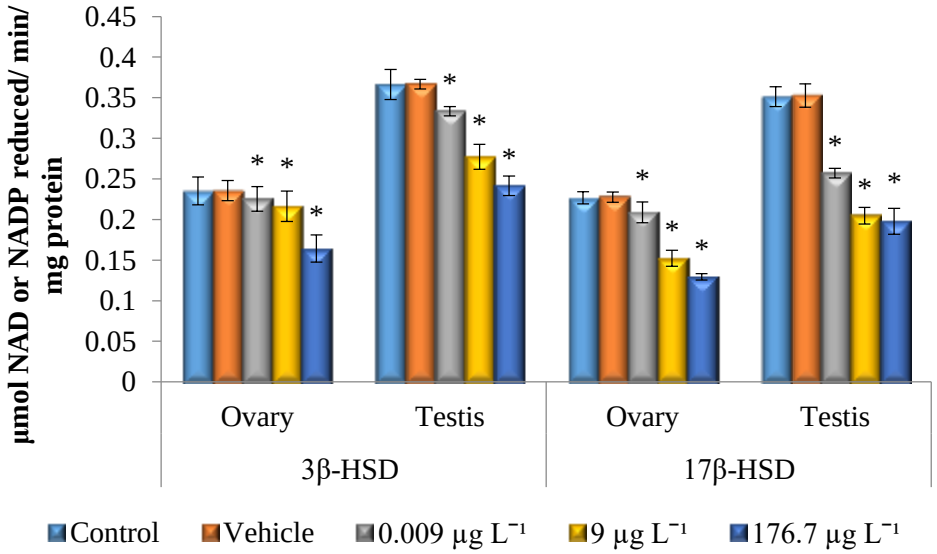

Fig. 6 **Effect of triclosan on the steroidogenic enzyme activities in the gonads of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)**

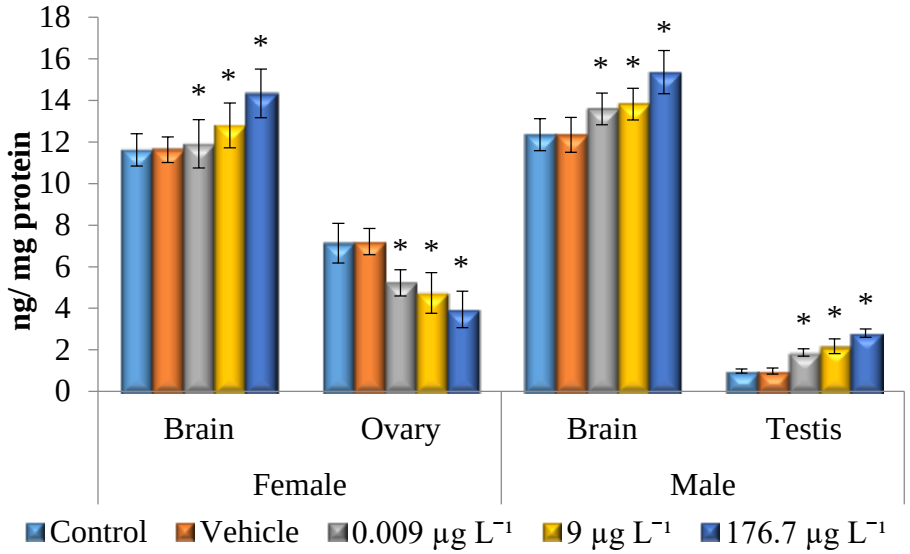

Fig. 7 Effect of triclosan on the concentration of aromatase enzyme in the brain and gonads of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)

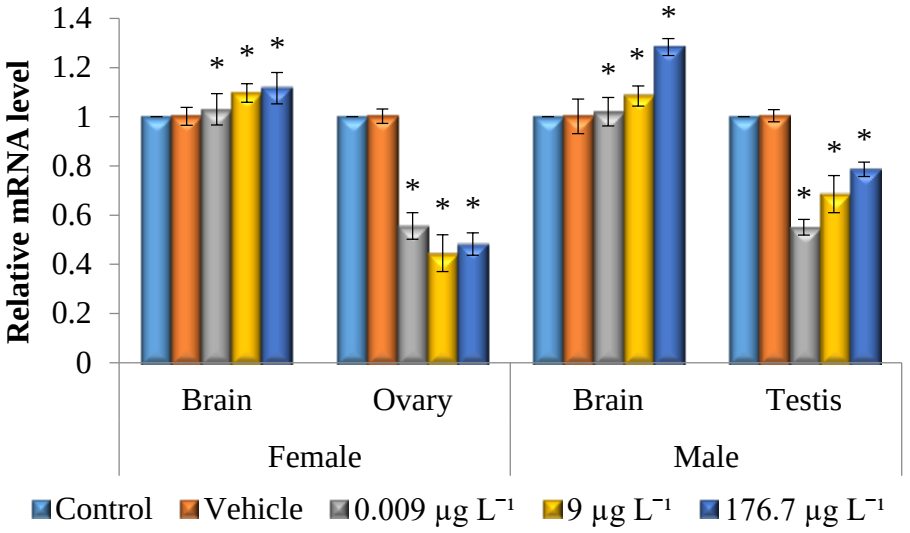

Fig. 8 Effect of triclosan on the aromatase gene (*Cyp19a1a*) in the brain and gonads of the fish *Anabas testudineus* (Mean ± SD; n = 3/ group, in replicates; Asterisks (*) denotes significant against the control groups)

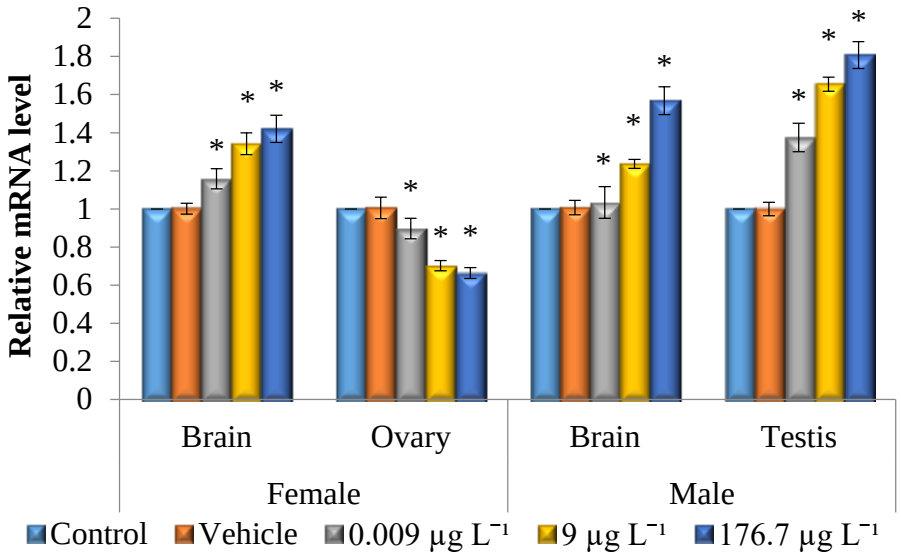

Fig. 9 Effect of triclosan on the aromatase gene (*Cyp19a1b*) in the brain and gonads of the fish *Anabas testudineus* (Mean ± SD; n = 3/ group, in replicates; Asterisks (*) denotes significant against the control groups)

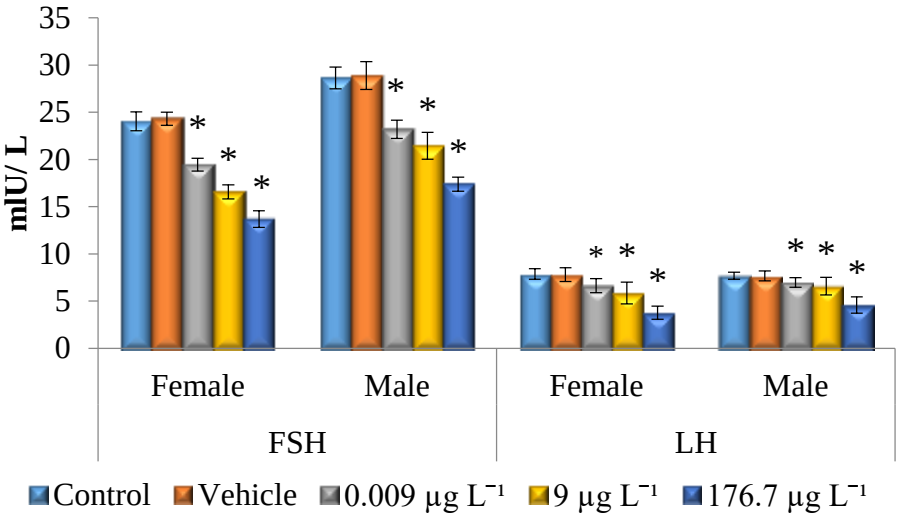

Fig. 10 Effect of triclosan on the levels of gonadotropic hormones in the serum of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)

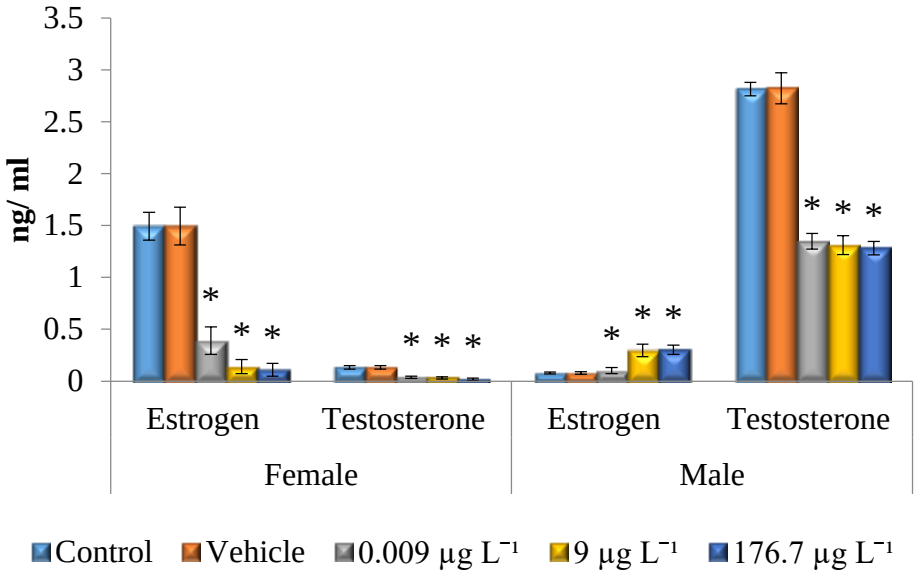

Fig. 11 Effect of triclosan on the levels sex steroid hormones in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Asterisks (*) denotes significant against the control groups)

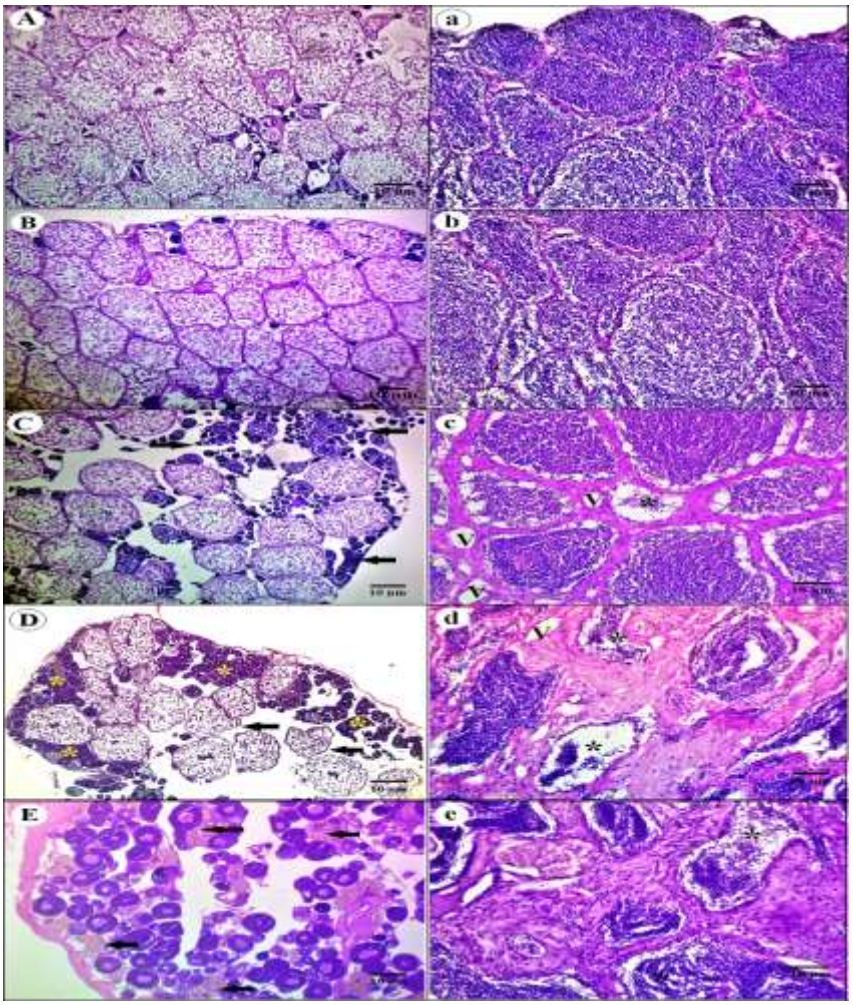

Fig. 12 Photomicrographs showing pre-spawning ovary (A-E) and testis (a-e) of the fish *Anabas testudineus*

A-Control; B-Vehicle showing vitellogenic oocytes; C-Triclosan at 0.009 µg L^{-1} for 90 d, loosely arranged oocytes (←); D-Triclosan at 9 µg L^{-1} for 90 d, loosely arranged oocytes (*), membrane blebbing (←); E-Triclosan at 176.7 µg L^{-1} for 90 d, melanomacrophage aggregation (←) ; a-Control; b-Vehicle showing compact seminiferous tubules; c, d, e-Triclosan-exposed groups (0.009, 9 and 176.7 µg L^{-1} for 90 d, respectively), Vacuolization (V), Loss of spermatozoa (*)

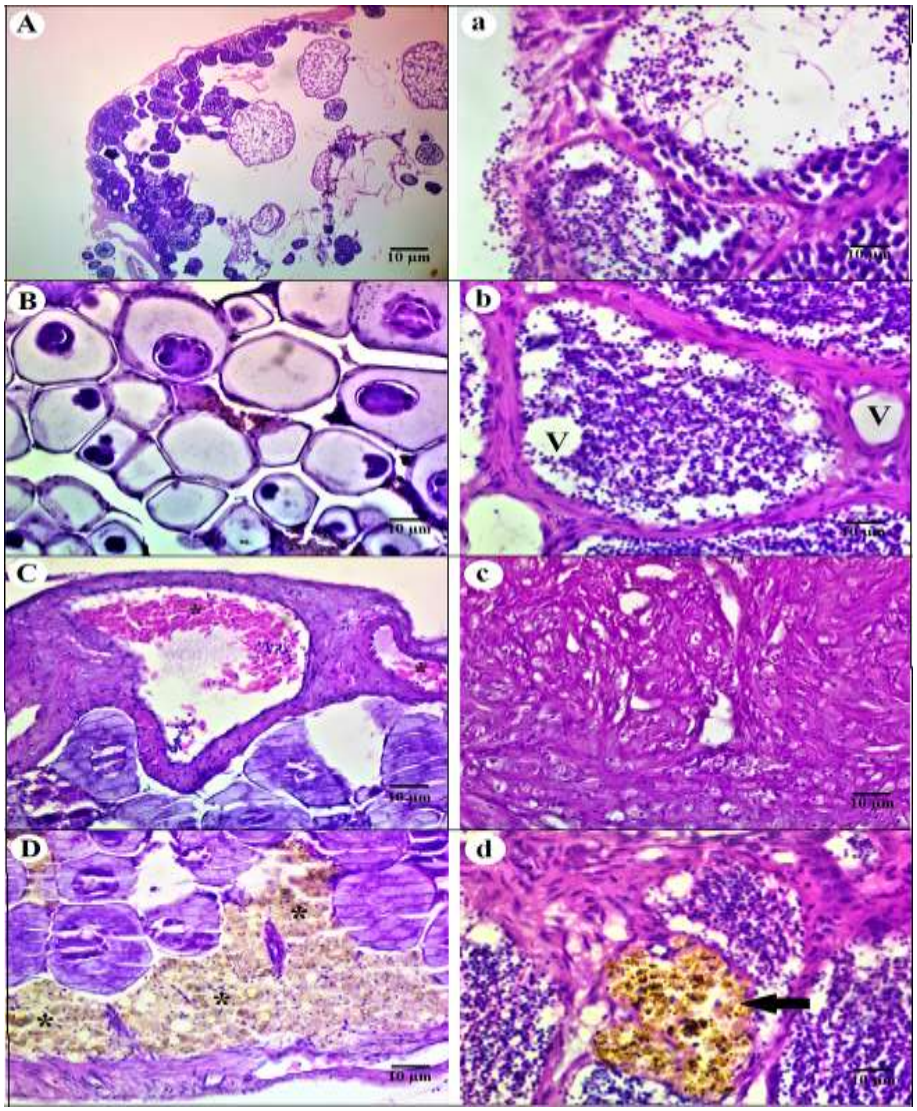

Fig. 13 Photomicrographs showing representative pathologies of pre-spawning ovary (A-D) and testis (a-d) in *Anabas testudineus*

A-Loosely arranged oocytes; B-Empty follicles; C-RBC infiltration (*); D-Melanomacrophage aggregation (*); a-Loss of spermatozoa; b-Vacuolization; c-differentiated seminiferous tubules; d-Melanomacrophage aggregation (←)

Table 1 Semi-quantitative scoring of pathological changes in the ovary of *Anabas testudineus* after triclosan exposure

Histological lesions	Triclosan		
	0.009 µg L^{-1}	9 µg L^{-1}	176.7 µg L^{-1}
Loosely arranged oocytes	+	++	+++
Membrane blebbing	+	++	+++
Aggregation of melanomacrophage centers	-	-	+++
Empty follicles	-	-	+++
RBC infiltration	-	-	+++
- Nil, + Mild, ++ Moderate, +++ Severe			

Table 2 Semi-quantitative scoring of pathological changes in the testis of *Anabas testudineus* after triclosan exposure

Histological lesions	Triclosan		
	0.009 µg L^{-1}	9 µg L^{-1}	176.7 µg L^{-1}
Vacuolization	+	++	+++
Thickening in the wall of seminiferous tubules	-	++	+++
Loss of spermatozoa	-	++	+++
Regression of seminiferous tubules	-	-	+++
Thickening of the interstitium	-	-	+++
Destruction of testicular epithelium	-	-	+++
Melanomacrophage aggregation	+	++	-
- Nil, + Mild, ++ Moderate, +++ Severe			

6. DISCUSSION

6.1 Effect of triclosan on the body weight, mucus deposition, and organ weights of the fish *Anabas testudineus*

Exposure of triclosan at 0.009, 9, and 176.7 µg L^{-1} concentrations for 90 d, during the pre-spawning period of *A. testudineus*, caused a significant reduction in the body weight in a concentration-dependent manner, in both sexes. Body weight is a valuable measure routinely used in toxicological studies to assess the treatment-related health status of the fish. The toxicants can enter into the body of fish through different routes such as oral, dermal, or operculum, and are suspected to cause serious disturbances in metabolism and feeding behavior. The reduction in the body weight of the fish after triclosan exposure indicated the direct toxic effects of the compound associated with anorexia or tissue damage. A similar reduction in the body weights has been observed after exposure of fullerene C_{60} nanomaterial to the fish, *A. testudineus* (Sumi and Chitra, 2020).

The mucus of the fish functions as a physiological barrier across the surrounding environment, and plays a key role in inter-and intra-specific chemical communication (Shepard, 1994). Teleosts consist of uniquely organized goblet, club, and sacciform cells in the skin and gill epithelial cells that contribute to the defense against the pathogens, or any toxic irritants (Groff, 2001; Salinas *et al*., 2011). Triclosan exposure increased the percentage of mucus deposition in *A.*

testudineus, which revealed the primary defensive mechanism of the fish to escape from the toxicant. Short-term exposure of triclosan to *Oreochromis niloticus* has been shown to increase mucus deposition in a time- and concentration-dependent manner (Vijitha *et al*., 2017). A similar stress-induced mucus deposition has been observed in the fish *A. testudineus* after exposure to fullerene C_{60} nanomaterial for 60 d and has been considered as the defense against the toxicant (Sumi and Chitra, 2019).

Organ weight analysis is often used as an endpoint in toxicology studies to identify the adverse effects of the toxicants. Absolute organ weight and organ to body weight ratio or relative organ weight provide accurate target-specific effects of the toxicant in the exposed organisms (Bailey *et al*., 2004). The Society of Toxicologic Pathology (STP) has recommended weighing of various tissues including brain, liver, gonads, etc., in multidose general toxicity studies as a relevant marker to evaluate target-specific toxicity of a compound (Sellers *et al*., 2007). The alterations in the brain weight have not been proved to be associated with neurotoxicity as the toxicants that alter the body weight do not always alter the brain weight (Wilson, 2001). The reduction in the brain weights after triclosan exposure at environmentally relevant concentrations and sublethal concentration revealed tissue-specific toxic effects of the toxicant.

In the present study, triclosan exposure caused a reduction in the absolute weights as well as in the gonadosomatic index (GSI) of gonads in a concentration-dependent manner in both sexes thereby

characterized treatment-related toxic effects. Evaluation of gonadal weight is the most valuable measure in sexually mature animals to interpret the reproductive toxicity of the tested compound (Tilton *et al*., 2005). Nonylphenol, an estrogenic compound exposed to Nile tilapia *Oreochromis niloticus* has been shown to decline the GSI, which was associated with histological alteration of ovary and decline in the levels of estradiol and vitellogenin in the female fish (Ali *et al*., 2014). Diuron and its metabolites exposed to male Nile tilapia have been shown to decrease the GSI and altered testicular histology with a profound reduction in the levels of testosterone and 11-ketotestosterone thereby suggested anti-androgenic activity of the herbicide (Pereira *et al*., 2015). The current observations clearly illustrated that the decline in female and male GSI with remarkable reproductive impairments suggests that triclosan possesses both estrogenic and anti-androgenic properties, similar to the phthalate plasticizers dibutyl phthalate (Aoki *et al*., 2011), and the herbicide tebuthiuron (de Almeida *et al*., 2018).

6.2 Effect of triclosan on the activities of gonadal steroidogenic enzymes

The activity of gonadal steroidogenic enzymes varies depending on species, sex, age, and reproductive stages where any alterations in the enzyme activities are used as an indicator of reproductive functions (Young *et al*., 2005). In the present study, the activities of the key steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase

(17β-HSD) evaluated in the pre-spawning stage of *A. testudineus* showed a concentration-dependent reduction in both ovary and testis of the fish. The findings suggested that triclosan disrupted gonadal steroidogenesis in the fish that ultimately lead to altered biosynthesis of gonadal hormones. The present results were in agreement with another study that sublethal exposure of dibutyl phthalate to the freshwater fish, *Pseudetroplus maculatus* decreased the activities of 3β-HSD and 17β-HSD in the ovarian and testicular tissues thereby suggested disruption in the gonadal steroidogenic pathway (Sajla *et al*., 2019; Sruthi *et al*., 2020).

6.3 Effect of triclosan on the concentration of aromatase enzyme in brain and gonads

Aromatase, the enzyme that converts testosterone into estradiol is associated with the maintenance of reproductive behavior (Cheshenko *et al*., 2008; Huffman *et al*., 2013), steroidogenesis (Tomy *et al*., 2007), and sexual differentiation in fish (Kwon *et al*., 2001). The present study observed that triclosan exposure elevated the concentration of aromatase enzyme in the brain and testis of *A. testudineus* indicating the auto-regulatory feedback loop driven by estradiol. However, the enzyme concentration was decreased in the ovary, and this could be due to the difference in the sensitivity of ovarian tissue towards the estrogenic compound. The concomitant decline in the level of estrogen in the female fish indicated failure of aromatization in the ovarian tissue. Further, the study observed that brain aromatase level was higher than in the gonadal tissues. The

current findings demonstrated that triclosan equally targeted brain and gonadal aromatase activity thereby revealing the endocrine-disrupting effects of the compound. Similar potential targets on the activities of the brain and gonadal aromatase enzyme have been documented after exposure of endocrine-disrupting chemicals in the zebrafish, *Danio rerio* (Hinfray *et al*., 2006). The inhibition in the aromatase concentration has been observed in the ovary of the marine fish cunner *Tautogolabrus adspersus* after exposure to environmental endocrine-disrupting chemicals such as estradiol and ethynylestradiol (Mills *et al*., 2014).

6.4 Effect of triclosan on the expression of aromatase (*cyp19a1a and cyp19a1b*) genes in brain and gonads

In teleosts, the aromatase genes namely *cyp19a1a* or aromatase A, and *cyp19a1b* or aromatase B are structurally and functionally different isoforms expressed in ovary and brain, respectively (Piferrer and Blazquez, 2005). Aromatase or estrogen synthetase is the member of the cytochrome P450 family involved in the biosynthesis of estrogen, and contributes to gonadal sex differentiation (Guiguen *et al*., 2010). The current study observed that triclosan exposure up-regulated *cyp19a1a* and *cyp19a1b* aromatase gene expression in the brain of both sex and testis, while down-regulated in the ovary thereby revealed a tissue-specific differential gene expression.

The up-regulated gene expression of *cyp19a1a* and *cyp19a1b* in the brain and testis of male fish suggest an increased estrogen synthesis during the pre-spawning period as evidenced by the rise in

the level of estrogen in serum. Estrogen is also involved in the neural aromatase expression through estrogen-responsive elements (EREs) in the promoter region of the *cyp19a1b* gene (Ramachandran *et al*., 1999). Besides, the presence of several putative cis-regulatory elements including an androgen-responsive element (ARE) found in the proximal promoter sequence of *cyp19a1b* genes makes the promoter sensitive to androgens as well (Sawyer *et al*., 2006; Diotel *et al*., 2010). However, the promoter region of *cyp19a1a* contains a steroidogenic factor-I (SF-1) regulatory element, which is less responsive to estrogen but activated by gonadotropins to increase *cyp19a1a* expression for estradiol production (Das and Mukherjee, 2013). The results were consistent with a previous study that showed an up-regulation of aromatase gene expression in the gonads and hypothalamus of juvenile male Yellow River carp, *Cyprinus carpio* exposed to triclosan, which has been mediated through the anti-androgenic mode of action (Wang *et al*., 2018).

In female fish, triclosan exposure caused up-regulation in gene expression of both aromatases in the brain while down-regulated in the ovary. The high affinity of triclosan towards the receptor of *cyp19a1a* gene, and its continuous exposure could have resulted in receptor saturation thereby down-regulated the gene expression in ovary (Li *et al*., 2017). Similar observation has been found in the ovary and brain tissues of zebrafish after bisphenol A exposure (Risalde *et al*., 2021). In teleosts, aromatase functions in the neural circuits to regulate behavior at the level of sensory perception, motor production, or a combination of these pathways to maintain social, breeding, and other

reproductive behavior (Shaw, 2018). Several studies have reported that triclosan exposure resulted in behavioral modifications like swimming alterations in *Pimephales promelas* and *Danio rerio* (Fritsch *et al*., 2013; Liu *et al*., 2018), aggression, and hyperactivity (Pullaguri *et al*., 2020). Aromatase expression is a good biomarker to test estrogen level and reproductive behavior (Guyon *et al*., 2012) where triclosan-induced modulation in the gene expression possibly affect the neurodevelopment resulting in behavioral modifications (Schultz *et al*., 2012). Thus the variability in aromatase expression observed in the current study serves as an indicator of reproductive impairment in the fish, *A. testudineus*.

6.5 Effect of triclosan on the levels of serum hormones

Steroidogenic enzymes play an important role in the biosynthesis of sex steroid hormones such as estradiol the female hormone, and testosterone the male hormone, which are mainly involved in germ cell development. Sex hormones act on the gonads for the maintenance of normal reproductive functions, and also act through feedback on the hypothalamus and pituitary for the regulation of the circulating levels of gonadotropins namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are involved in embryonic development, sex differentiation, metabolism, behavior, and reproduction (Tokarz *et al*., 2015).

The present study determined the serum levels of FSH, LH, estrogen, and testosterone in both sexes after triclosan exposure, and was compared with the respective control groups. Triclosan exposure

to the female fish during the pre-spawning period of reproduction caused a concentration-dependent decrease in the levels of serum FSH, LH, estrogen, and testosterone. The results suggested that triclosan affected sex hormone production by disrupting the activity of ovarian steroidogenic enzymes. Besides, inhibition in the aromatase enzyme activity in the ovary by triclosan could have prevented the conversion of androgen to estrogen thereby reduced the levels of sex hormones in female fish. The decline in the circulating levels of gonadotropins such as FSH and LH after triclosan exposure revealed that the endocrine-disrupting effects of the toxicant were mediated through the negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis that finally lead to the decline of estradiol (Redding and Patino, 1993). In normal conditions, the stimulation of the hypothalamus releases gonadotropin releasing hormone (GnRH) for the secretion of gonadotropin, which was prevented by the endocrine disrupting effect of triclosan thereby disrupted the normal hormonal cycle.

Exposure of triclosan to the male fish in the pre-spawning period caused a decrease in the levels of serum FSH, LH, and testosterone while the level of estrogen increased in a concentration-dependent manner. The inhibition in the testicular steroidogenic enzymes after triclosan exposure had down-regulated the production of testosterone, and also inhibited the gonadotropin secretion. The anti-androgenic effects of triclosan were evident by the decrease in testosterone level by blocking the negative feedback either at the hypothalamus or pituitary thereby regulated the production of GnRH or gonadotropins, respectively. Therefore, triclosan-induced reduction

in FSH and LH levels combined with a decline in testosterone indicated reproductive toxicity in the male fish.

Meanwhile, triclosan exposure increased the estrogen level in male fish and this could be due to the increase in aromatase enzyme activity by interfering with the male reproductive axis, which indicated the potential non-estrogen receptor-mediated anti-androgenic effects of the toxicant. A similar mechanism of hormonal imbalance has been reported in the juvenile male Yellow river carp *Cyprinus carpio* proving anti-androgenic effects of triclosan in male fish (Wang *et al*., 2018). The auto-regulatory loop involving estradiol production from testosterone by enhancing the activity of brain aromatase can lead to low sperm counts and decreased sperm quality, which is suspected to affect male fertility in fish (Tang *et al*., 2017).

6.6 Effect of triclosan on the histology of gonads

Histological examination is a reliable biomarker of stress to evaluate the health status of fish (Van der Oost *et al*., 2003). The present study examined the effect of triclosan on histological aspects of gonadal tissue in *A. testudineus* in the pre-spawning period of reproduction. Triclosan exposure at varying concentrations showed severe lesions in the ovary such as loosely arranged oocytes, formation of membrane blebbing as a sign of oocyte regression, absence of vitellogenic oocytes stating reduced ovarian maturity. The ovarian follicles predominantly consisted of the developing chromatin nucleolus and perinucleolus stages with empty follicles, RBC infiltration and aggregation of melanomacrophage centers. The

findings illustrated that triclosan exposure caused a shift of ovarian tissue towards various stages of degeneration in the pre-spawning phase that eventually lead to failure of spawning and reproductive functions.

Triclosan exposure increased the number of immature, regressed and loosely arranged oocytes due to interstitial tissue damage, which was found associated with the reduction in gonadosomatic index of female fish. In another study, zebrafish exposed to estrogenic chemicals have been shown to cause loss of mature ovarian follicles, and atresia thereby suppressed gonadal development (Weber *et al*., 2003). Similarly, sublethal exposure of dibutyl phthalate has been observed to induce morphological changes in the ovary as evident by the formation of atretic oocytes, empty follicles, membrane blebbing, vacuolization, nuclear condensation, and broken theca granulosa membrane in the fish *Pseudetroplus maculatus* (Sajla *et al*., 2019).

Exposure of triclosan also caused remarkable histomorphological changes in the testis including vacuolization, regressed and thickened wall of seminiferous tubules, loss of spermatozoa, thickened interstitium, and formation of melanomacrophage aggregation. The present result is consistent with another study in which exposure of anti-androgenic chemicals such as flutamide, bisphenol A, and octylphenol to guppy *Poecilia reticulata* at varying concentration altered the architecture of testis such as thickened interstitium, loss of tubular structure in seminiferous tubules, increase in the interstitial area, loss of Sertoli and Leydig cells (Kinnberg and Toft, 2003). Similarly, in another study flutamide

exposed to Murray rainbowfish, *Melanotaenia fluviatilis* affected testicular development with an increase in the proportion of spermatocytes having lack of spermatogonia and spermatozoa in the testis (Bhatia and Kumar, 2016).

Despite this, a reduction in the gonadosomatic index of male fish observed after triclosan exposure demonstrates the testicular damage and negative impact on male reproduction. Thus triclosan-induced adverse effects in male gonads also contributed to the disruption in gonadal development and steroidogenesis in the fish, *Anabas testudineus*. Briefly, this study provides direct evidence that triclosan possesses both estrogenic and anti-androgenic properties to cause reproductive impairment in the fish *A. testudineus*.

7. CONCLUSIONS

1. Exposure of *Anabas testudineus* to triclosan at environmentally relevant and sublethal concentrations for 90 d during the pre-spawning period caused reduction in the body weight, and weights of brain and gonadal tissues along with an increase in mucus deposition stating the adverse effects of the toxicant.
2. Triclosan declined the activities of the key steroidogenic enzymes namely 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) in ovary and testis suggesting disruption in the gonadal steroidogenic pathway.
3. Triclosan exposure elevated the concentration of aromatase enzyme in the brain and testis of the fish through the auto-regulatory feedback loop driven by estradiol. However, the enzyme activity was decreased in the ovary, which revealed difference in the sensitivity of ovarian tissue.
4. In female fish, triclosan declined the levels of serum FSH, LH, estrogen, and testosterone mediated through multiple pathways as disruption in ovarian steroidogenic enzymes, and inhibition of aromatase enzyme mediated through the negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis.
5. In male fish, decrease in the levels of gonadotropins and testosterone with an increase in the level of estrogen indicate anti-androgenic effects of triclosan by affecting the feedback at HPG axis.

6. Up-regulation of aromatase gene expression in the gonads and brain of male fish demonstrated the anti-androgenic mode of action while up-regulation in the brain and down-regulation in the ovary illustrated estrogenic effects of triclosan in female fish.
7. Triclosan exposure at environmentally relevant and sublethal concentrations caused severe lesions in the ovary and testis during the pre-spawning phase of reproduction by disrupting the gonadal development and steroidogenesis, which could affect spawning, gamete quality, and fertility thereby suspected to cause reproductive impairment in the fish *A. testudineus*.

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CHAPTER 5

Influence of triclosan on the reproductive physiology of *Anabas testudineus* during the spawning phase

Chapter 5 **Influence of triclosan on the reproductive physiology of *Anabas testudineus* during the spawning phase**

CONTENTS

LIST OF ABBREVIATIONS

°C	Degree Celsius
µg L^{-1}	Microgram per Litre
µm	Micrometers
µM	Micromol
11-KT	11 ketotestosterone
17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
ANOVA	Analysis of Variance
d	days
DMSO	Dimethyl sulfoxide
DPX	Dibutylphthalate Polystyrene Xylene
E2	Estradiol
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
g	Relative Centrifugal Force
GnRH	Gonadotropin-releasing hormone
GSI	Gonadosomatic index
GTH	Gonadotropin
HPG	Hypothalamus-pituitary-gonadal
HPGL	Hypothalamic-pituitary-gonadal-liver
HPT	Hypothalamus-pituitary-thyroid
KDa	Kilo Dalton
L	Litre
LH	Luteinizing hormone
LLTP	Large Lipid Transfer Protein
mg	Milligram
mg L^{-1}	Milligram per litre
min	Minutes

ml	Millilitre
mM	Millimolar
MVB	Multi vesicular bodies
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
ng	Nanogram
nm	Nanometer
OECD	The Organisation for Economic Co-operation and Development
PPCPs	Pharmaceuticals and Personal Care Products
ppm	Parts per million
rpm	Rotation per minute
StAR	Steroidogenic acute regulatory gene
T_3	3,5,3'-triiodo-L-thyronine or triiodothyronine
T_4	L-thyroxine
THR-A	Thyroid hormone receptor α gene
THR-B	Thyroid hormone receptor β gene
TR	Thyroid hormone receptors
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
v/v	Volume per volume

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1. INTRODUCTION

1.1 Physiology of female reproduction in teleosts

The female reproductive physiology of teleosts fishes comprises a hollow-sac-like ovary consisting of numerous ovigerous folds lined by germinal epithelium. The ovary possesses germ cells that produce haploid gametes, granulosa cells involved in the production of steroids, and theca cells which provide structure and support to the maturing follicles. Germ cells are endodermal in origin, which divide meiotically, and are then arrested at the prophase stage of the first meiotic division to transform into non-yolky primary oocytes (Nagahama and Yamashita, 2008). The granulosa cells and theca cells are commonly referred to as the somatic cells, which construct the ovarian capsule, ovarian follicles, and interstitial cells innervated by neurovascular tissues.

On the basis of the pattern of ovarian capsule formation, two types of the ovary namely cystovarian and gymnovarian were found prominent in teleost fishes. The cystovarian type is a saccular structure surrounded by an ovarian capsule with a central lumen where the internal walls are formed of several irregular folds or cell lamellae that project into the lumen. In the pre-vitellogenesis and vitellogenesis stages, the lamellae consisting of supporting tissue or stroma surrounds the developing follicles. This type of ovary is commonly found in most teleosts including goldfish, tilapia, guppies, etc. (Urbatzka *et al*., 2011).

The gymnovarian type lacks ovarian capsule and ovocoel so the ovulated eggs are discharged into the abdominal cavity, and then spawned through the genital pore, and this type was familiar in salmonids and eels (Kagawa, 2013).

The development of primary oocytes from the primordial germ cells occurs through a series of mitotic and meiotic events. Later, the accumulation of yolk in the developing oocytes progresses to the oocyte maturation (Devlin and Nagahama, 2002). The process of oocyte maturation is a complex cellular event comprised of germinal vesicle breakdown, condensation of the chromosome, assembly of the meiotic spindle, and formation of the first polar body. The mature eggs are spawned at the metaphase II stage and the polar body is released following the fertilization. The developing oocytes are surrounded by two layers of cells namely inner continuous granulosa cells with the basement membrane, and outer theca cells, which are collectively known as follicular envelopes (Nagahama, 1983). The granulosa layer is formed of micropyle cells, whereas theca layer consists of collagen fibers, blood vessels, fibroblasts, and steroid-producing cells (Guraya, 1986; Le Menn *et al*., 2007).

During gametogenesis, the primary growth phases of the ovary are represented by chromatin nucleolus and perinucleolus stages, which are together referred to as the preparatory stage (Le Menn *et al*., 2007). The secondary growth phase is the vitellogenesis stage where the biosynthesis, processing, and deposition of hepatic yolk protein called vitellogenin occurs (Le Menn *et al*., 2007). The primary vitellogenic stage of the developing oocytes is identified by the

presence of yolk vesicle or cortical alveoli, oil droplets, and yolk globules (Selman *et al*., 1993). Along with the proportional increase in the size of oocyte, the yolk vesicle consisting of enormous mucopolysaccharides, triglycerides, or glycoprotein also increases and are displaced into the periphery of the oocyte cytoplasm to form cortical alveoli (Lubzens *et al*., 2010).

Vitellogenin (Vtg), a phospholipoglycoprotein, is predominantly synthesized in liver tissues, and also in some non-hepatic tissues such as the intestine and ovary, where it is regulated by estrogen (Babin *et al*., 2007; Wang *et al*., 2005). Vitellogenin is a large protein that belongs to the Large Lipid Transfer Protein (LLTP) superfamily, whose amino acid sequence is arranged into different domains and each represents different yolk proteins or lipovitellins, which are stored as yolk globules in the developing oocytes. Lipovitellins exist in different forms in teleosts oocytes like VtgA, VtgB, and VtgC (Babin *et al*., 1999).

Vitellogenin easily passes through the granulosa and theca layers of the oocyte, opening the pore canals of the zona radiata by the process of receptor-mediated endocytosis to enter into the ooplasm, where it fuses with lysosomes to form multivesicular bodies (MVB) (Wallace and Selman, 1990). Later, the lysosomal enzyme known as cathepsin D cleaves the MVB into the smaller yolk proteins (Sire *et al*., 1994; Carnevali *et al*., 1999). The development of the covering of the egg or vitelline envelope continues along with the development of oocytes till it attains maturity. Then the egg envelops get differentiated into inner zona radiata interna and outer zona radiata externa (Liu *et al*.,

2006; Modig *et al*., 2007). A prominent antibacterial property is found in the egg envelope to protect against invasive microorganisms. However, after fertilization, the egg envelop develops to become a thick chorion layer, which protects the egg in the aquatic environment (Modig *et al*., 2007).

1.2 Physiology of male reproduction in teleosts

Teleosts testis are paired organs covered by tunica albuginea, which encloses interstitial or intertubular sections and tubular compartments. The interstitial section is continuous with the tunica albuginea, and it contains Leydig cells, which are bathed by blood and lymphatic vessels, macrophages, mast cells, neural and connective tissue (Koulish *et al*., 2002). The tubular compartment consists of germ cells including spermatogonia, spermatocytes, spermatids, and spermatozoa along with the somatic Sertoli cells. Testis performs two main functions namely spermatogenesis and steroidogenesis, which are effectively involved in the production of spermatozoa and steroid hormones, respectively. The interaction of Sertoli cells with the developing germ cells determines the spermatogenic capacity of a testis (Matta *et al*., 2002).

Spermatogenesis is a developmental process that transforms undifferentiated diploid spermatogonia into highly specialized haploid spermatozoa that occurs in three phases namely the spermatogonial phase, meiotic phase, and spermiogenesis. Fish exhibits a cystic mode of spermatogenesis where the cytoplasmic extension of Sertoli cells forms a cyst that encloses the developing group of germ cells derived

from a single spermatogonium (Callard, 1996). The cystic Sertoli cells are more efficient in supporting the developing germ cells as it provides specific growth factors for the development, and also yield germ cells with a low rate of apoptosis (Schulz *et al*., 2005). Based on the distribution of spermatogonia in the germinal compartment, the testis is classified into two types namely restricted and unrestricted spermatogonial distribution (Grier, 1981; Schulz *et al*., 2010).

The restricted spermatogonial distribution is found common in higher teleosts of the orders Atheriniformes, Beloniformes, and Cyprinodontiformes. In this type, the Sertoli cells found near the tunica albuginea surround the undifferentiated spermatogonia, and when it enters the meiotic phase, the cyst migrates towards the spermatic duct for spermiation (Parenti and Grier, 2004). The unrestricted distribution of spermatogonia is a primitive pattern found common in the orders like Cypriniformes, Characiformes, and Salmoniformes (Parenti and Grier, 2004). In this type, spermatogonia are widely distributed throughout the testis, where the cysts do not move or displaced during the development (Grier, 1981). Besides, an intermediate form of spermatogonial distribution is also found in some teleosts like Perciformes, Pleuronectiformes, or Gadiformes (Vilela *et al*., 2003; Garcia-Lopez *et al*., 2005; Almeida *et al*., 2008). In this type, spermatogonia are found close to the periphery in the early stages of development while moving close to the spermatic duct during the advanced stages by the cyst-generating activity of the germinative zone (Almeida *et al*., 2008).

1.3 Hypothalamic-pituitary-gonadal-liver (HPGL) axis

Reproductive processes and strategies are regulated by the interplay of hormones released from the hypothalamus, pituitary, and gonads, which are referred to as the hypothalamus-pituitary-gonadal (HPG) axis (Yaron *et al*., 2003). In fish, the liver plays a significant role in the regulation of gonadal differentiation, maturation, and other reproductive processes of females, which are referred to as the hypothalamus-pituitary-gonadal-liver (HPGL) axis. The preoptic area of the hypothalamus synthesizes and releases the neuropeptide hormone namely the gonadotropin-releasing hormone (GnRH). It directly binds to the specific receptors in the gonadotropic cells of the anterior pituitary and stimulates to produce the gonadotropins (GTH) such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the circulation to target the gonadal tissues. The secretions of GnRH and gonadotropins are largely modulated by the action of monoamine neurotransmitters like dopamine and serotonin (Thomas, 2008).

The two major gonadotropic hormones or gonadotropins such as FSH or GTH-I, and LH or GTH-II are involved in fish reproduction. Gonadotropins are glycoprotein hormones of approximately 28 to 30 KDa molecular weights, composed of two subunits including a common alpha subunit (α-FSH or α-LH), and a specific beta subunit (β-FSH or β-LH) produced from different gonadotropic cells of the anterior pituitary. The gonadotropins act through the specific G-protein-coupled receptors i.e., FSH-R or LH-R on follicle cells of the

ovary or Leydig cells of the testis. Signal transduction occurs through adenylyl cyclase, and calcium-dependent second-messenger signaling pathways for the regulation of spermatogenesis or steroidogenesis process in fish (Thomas, 2008).

Ovary responds to the stimulus of gonadotropins to secrete the most potent female sex steroid hormones like 17β-estradiol that are involved in oogenesis, vitellogenesis, and ovulation. However, the testis responds to the stimulus of gonadotropins, and secretes the male sex steroid hormones like androgens, mainly 11 ketotestosterone (11-KT) to regulate spermatogenesis and spermiogenesis (Yaron and Levavi-Sivan, 2011).

During the process of oogenesis, oogonial proliferation and meiosis are initiated by the estradiol, synthesized by the FSH surge. The vitellogenin or yolk protein, and choriogenin or chorionic protein of hepatic origin are known to sequester and deposits in the developing oocytes under the influence of estrogen hormone. The production of estrogen, the process of vitellogenesis, and sequestration of vitellogenin in oocytes are regulated by FSH. The synchronous ovaries of teleost fishes show distinct phases such as pre-vitellogenic, vitellogenic, and post-vitellogenic oocytes regulated under the influence of gonadotropins (Yaron and Levavi-Sivan, 2011).

Spermatogenesis is a complex process characterized by three major phases namely mitotic proliferation of the spermatogonia, the meiotic division of the primary spermatocyte, and the conversion of the haploid spermatids into flagellated spermatozoa (Nagahama *et al*., 2007). Environmental cues like photoperiod and temperature also

influence the hypothalamus-pituitary-gonad axis (HPG) to release gonadotropin-releasing hormone (GnRH) from the hypothalamus. The GnRH stimulates the pituitary to release gonadotropins such as FSH and LH for the process of spermatogenesis and testicular steroidogenesis, by acting on the receptors of Sertoli cells (FSH-R) and Leydig cells (LH-R), respectively, under the influence of 11-ketotestosterone (Miura and Miura, 2003). Besides, during the spermiation process, the receptors of progestins in the membrane and nucleus are highly expressed to regulate several testicular functions including sperm maturity, milt production, (Yueh and Chang, 1997), and sperm motility (Tubbs and Thomas, 2008).

1.4 Role of hypothalamus-pituitary-thyroid (HPT) axis in reproduction

Thyroid hormones play an important role in several physiological processes including differentiation, development, growth, osmoregulation, metabolism, metamorphosis, and reproduction in fish (Jia *et al*., 2016). The hypothalamus-pituitary-thyroid axis (HPT) influences the synthesis, secretion, transport, and metabolism of thyroid hormones (Fliers *et al*., 2014). The neurosecretory cells of the hypothalamus secrete thyrotropin-releasing hormone (TRH), which stimulates the thyrotrope cells of the anterior pituitary gland for the secretion of thyroid-stimulating hormone (TSH), which in turn act on the thyroid follicles to secrete thyroid hormones (De Groef *et al*., 2006). TSH is a heterodimeric protein with identical α subunits, and functionally and structurally distinct β subunits (Yoshiura *et al*., 1999).

Thyroid follicles are the functional unit of thyroid systems consisting of specialized secretory cells named thyrocytes that produce thyroglobulin, a glycoprotein into the lumen. The iodinated tyrosine, the precursor amino acid under the influence of TSH forms 3,5,3'-triiodo-L-thyronine or triiodothyronine (T_3), and L-thyroxine or T_4 in the thyroid follicles (Blanton and Specker, 2007). The thyroid hormone receptors (TR) such as TRα and TRβ encoded by *THR-A* and *THR-B* genes are transcribed to numerous isoforms like TRα1, TRα2, TRβ1, and TRβ3 (Tovo-Neto *et al*., 2018). The synthesis and secretion of thyroid hormone are controlled by a negative feedback loop, where the plasma T_3 and T_4 levels regulate the release of TRH or TSH at the hypothalamus or pituitary through the HPT axis (Yoshiura *et al*., 1999; Zhang *et al*., 2018).

In some teleost fishes, the receptors of TSH are identified in oocytes and testicular cells beyond the thyroid follicles indicating the direct involvement of thyroid hormone in reproduction (Kumar *et al*., 2000). The thyroid hormone receptors show differential expression in testes and ovaries where high expression occurs in the ovary from the early gonadal development till spawning period but remained low during the process of vitellogenesis (Goto-Kazeto *et al*., 2003). Similarly, in males thyroid hormones are involved in spermatogenesis by promoting germ cell proliferation and differentiation, and also stimulate androgen production, testicular differentiation, and development through the regulation of the Sertoli cell (Tovo-Neto *et al*., 2018).

1.5 Role of endocrine disruptors in intersex formation

The presence of male and female gonads together in the same individual is referred to as intersex formation, where it results in feminization or masculinization (Tyler and Jobling, 2008). Feminization refers to the presence of oocytes in the testes (Nolan *et al*., 2001), and masculinization means the occurrence of the testis with spermatozoa in the oocytes (Hinck *et al*., 2007). The intersex condition in fish is known in different terminology as testicular oocytes, testicular follicles, testis-ova, ovotestis, etc. (Blazer *et al*., 2011). Ovo-testis is a condition of mature ovarian tissue interspersed with testicular tissue, while testis-ova denotes the testis with developing ovarian follicles, or oocytes within a mature testis (Getsfrid *et al*., 2004). The intersex in fish occurs due to the adverse effects of endocrine-disrupting chemicals in the aquatic ecosystem.

The consequences of intersex in teleosts are found associated with the assessment of some reproductive parameters such as gonadosomatic index (GSI), vitellogenin production, and levels of sex steroid hormones. GSI is the relative weight of gonads proportional to the body weight, which is often estimated as an indicator of the reproductive health of the fish. It is a useful endpoint to assess gonadal growth, sexual maturation, reproductive strategies, and reproductive stages of the fish (Chiang *et al*., 2012). Besides, GSI is used to assess the effects of endocrine disruptors in the aquatic environment. It was demonstrated that the variation in gonad size is more consistent with the presence of stressors in the site than the intersex formation (Bahamonde *et al*., 2013).

Vitellogenin, the egg yolk protein synthesized in the female liver reaches the developing oocytes for nourishing the embryo. Male fishes normally lack the expression of the vitellogenin gene as it is regulated by estradiol, the female hormone. The exposure of endocrine disruptors increases the level of vitellogenin in males and influences the formation of intersex or feminization (Sole *et al*., 2003). Sex steroids play an important role in sex differentiation, gonadal maturation, and maintenance of various reproductive strategies in fish. Endocrine disruptors disrupt the levels of sex steroid hormones and consequently increase the ratio of intersex in the population (Hinck *et al*., 2009). EDCs possessing an estrogenic or anti-androgenic mode of action facilitate the formation of intersex mediated mainly by disrupting the HPG axis. However, molecular methods such as transcriptomics and proteomics determine the specific pathways that lead to intersex in the fish population (Bahamonde *et al*., 2013).

1.6 Gonadal histopathology - a biomarker for endocrine disruption

Histopathology is a semi-quantitative microscopic examination of cells or tissues of an organism to evaluate the effects of aquatic contaminants than any single biochemical parameter (Martins *et al*., 2016). Fish histology provides comprehensive information to understand the relationship between the concentration of toxicants in the environment and its effects on the target organs. Gonadal histopathology serves as a diagnostic endpoint to understand and evaluate the effects of potential endocrine-disrupting chemicals in the

fish. Most of the EDCs mainly target gonadal tissues at different stages of reproduction thereby interfere with egg production in females, and sperm production in males.

The prominent histological alterations that occur in the ovary of the fish includes atretic or regressed follicles, disruption in follicular epithelial cells, vacuolization of oocytes, formation of interfollicular spaces, empty follicles, nuclear condensation, disruption of yolk vesicles, clumping of cytoplasm in maturing oocytes, and stromal hemorrhage (Miles-Richardson *et al*., 1999; Lange *et al*., 2001; Opute *et al*., 2021). The primary histological alterations identified in testis include vacuolization of germ cells, formation of melanomacrophage aggregates, presence of testicular oocytes, pyknosis, dilation, and disorganization of seminiferous tubules, reduced or absence of spermatozoa (Leino *et al*., 2005; Qiang and Cheng, 2021). The developmental defects of the gonadal tissues contribute to understanding the functional relationships on reproduction of the fish. Therefore, histological changes are served as a biomarker to examine specific target organs, and also determine the health status and fitness of an organism in the aquatic ecosystem.

1.7 Relevance of the study

The spawning phase of fish reproduction is the most sensitive and precise stage that exhibits several physiological modifications for the successful release of eggs into the aquatic environment, which also determines the reproductive fitness or success to retain the population. The endocrine-disrupting effects of triclosan and its involvement in

reproductive toxicity mediated through different modes of action in various fish species have been documented. The present study was aimed to explore the hypothesis that triclosan at environmentally relevant and sublethal concentrations could influence the reproductive physiology of the fish, *Anabas testudineus* during the spawning phase. The assessment of some specific reproductive endpoints such as gonadosomatic index, vitellogenin level, gonadal steroidogenic enzymes, fecundity, sperm indices, and gonadal histology provides an indication to confirm the reproductive toxicity of triclosan in the spawning phase of the fish.

2. OBJECTIVE OF THE STUDY

- To understand the Influence of triclosan on the reproductive physiology of *Anabas testudineus* during the spawning phase

3. REVIEW OF LITERATURE

Triclosan, a major ingredient found in many antimicrobial consumer products, has been known to drain from domestic untreated wastewater effluents to the aquatic environment, and also bioaccumulate in aquatic species. The antimicrobial property of triclosan targets microbial pathogens hence it is intentionally used as a biocide for a short period during hand washing, however, when it is released into the environment could harm the non-target organisms, including fish in the aquatic ecosystem. Triclosan is a lipophilic compound that shows functional similarity with some known non-steroidal estrogenic chemicals such as polychlorinated biphenyls, polybrominated diphenyl ethers, diethylstilbestrol, dioxins, bisphenol A, etc., and also shares structural similarity with the thyroid hormone thereby cause potential disruption to the endocrine system of both aquatic and terrestrial vertebrates, including humans (Ishibashi *et al*., 2004). The World Health Organization (WHO) has identified triclosan as the potential endocrine-disrupting chemical since it shows a high affinity to interact with the endocrine system (WHO-UNEP, 2012). Many literatures have addressed that triclosan exposure resulted to interrupt many endocrine-related processes including developmental defects, imbalance in hormone homeostasis, and reproduction.

Exposure of triclosan to the early life stages of medaka, *Oryzias latipes* delayed hatchability rate and increased hepatic vitellogenin concentration showing weak estrogenic effects (Ishibashi

et al., 2004). The embryotoxicity of triclosan in early stages of zebrafish has been documented in the form of hatching delay, while in adults it caused alterations in biomarker enzymes such as cholinesterase, glutathione S-transferase, and lactate dehydrogenase (Oliveira *et al*., 2009). The ovo nano injection of triclosan with other pharmaceutical products such as diclofenac and carbamazepine to Japanese medaka, *Oryzias latipes* has been shown to affect the survival of embryos along with a delay in embryonic development, heartbeat rate, hatchability, hatching time of embryos, and upward swimming of larvae suggesting the possible uptake of the toxicant from maternal extragonadal tissues (Nassef *et al*., 2010). The endocrine-disrupting effects of triclosan have been demonstrated in the male mosquitofish, *Gambusia affinis* as evident by an increase in the hepatosomatic index, hepatic vitellogenin gene expression with a reduction in sperm counts (Raut and Angus, 2010).

Triclosan exposed to fathead minnow *Pimephales promelas* altered swimming behavior and nest defense behavior along with alterations in other endpoints of development such as growth, and escape performance with changes in the histology of liver and gonads suggesting developmental and reproductive toxicity (Schultz *et al*., 2012). In a study, laccase of *Coriolopsis gallica* has been used for the transformation of estrogenic compounds like estradiol, nonylphenol, bisphenol A, and triclosan into their polymers, which observed an efficient elimination of the compounds having estrogenic activity, particularly triclosan and bisphenol A by reducing the effects of estrogen (Torres-Duarte *et al*., 2012).

Dietary exposure of triclosan to zebrafish resulted in hyperplasia of thyroid follicles and caused up-regulation of the sodium-iodide symporter and thyroid-stimulating hormone coupled with reduced thyroid gland activity demonstrating adverse effects on fish thyroid axis (Pinto *et al*., 2013). Adult fathead minnows *Pimephales promelas* exposed to triclosan for 48 h has been shown to upregulate the level of hepatic vitellogenin in females and males along with hepatic lipoprotein lipase suggesting estrogenic effects of the compound (Zenobio *et al*., 2014).

Exposure of triclosan to the sheepshead minnow, *Cyprinodon variegatus* has disrupted the thyroid system by altering the concentration of T3 in the thyroid axis that ultimately resulted in developmental delay, metamorphosis, and reduction in juvenile fitness (Schnitzler *et al*., 2016). Zebrafish *Danio rerio* exposed to triclosan exhibited toxic effects by interacting with thyroid hormone receptor β and aryl hydrocarbon receptor through the induction in the transcription of thyroid hormone-associated genes that lead to altered the activities of phase I and phase II enzymes (Zhou *et al*., 2017).

In a study, Smallmouth bass *Micropterus dolomieu* sampled from the St. Joseph River in northern Indiana with a quantifiable level of triclosan has explored the presence of testicular oocytes in the spawning season, and altered expression of gonadal and liver genes involved in sex differentiation and reproductive functions namely *esr1*, *esr2*, *foxl2*, *fshr*, *star*, *lhr*, and *vtg* suggesting the formation of gonadal intersex (Abdel-Moneim *et al*., 2017). In the female and male Yellow River carp, *Cyprinus carpio* under semi-static condition for 42 d,

triclosan exerted vitellogenin induction through the hypothalamic-pituitary-gonadal (HPG) axis (Wang *et al*., 2017; Wang *et al*., 2018). The effects of triclosan on the reproduction in teleost fish species have been investigated during the early life stages using Japanese medaka *Oryzias latipes* as the laboratory model, which revealed a reduction in the fecundity rate along with alteration in liver vitellogenin concentration in both sexes, however, the fertility rate was not affected (Horie *et al*., 2018).

A study conducted in Lake Mead national recreation area in the Southwestern United States contaminated with methyl triclosan along with other chemicals such as polychlorinated biphenyls, hexachlorobenzene, and galaxolide has reported alteration in gamete quality and endocrine- and reproductive defects in male common carp *Cyprinus carpio*, as evident by the alteration in sperm quality parameters such as motility, viability, mitochondrial membrane potential, sperm count, sperm morphology, and DNA fragmentation along with significant modifications in the plasma components like vitellogenin, 17ß-estradiol, 11-keto-testosterone, triiodothyronine, and thyroxine levels (Jenkins *et al*., 2018).

Long-term exposure of triclosan to adult zebrafish *Danio rerio* from 6 to 90 d post-fertilization has been shown to cause deleterious effects on liver growth and development. The study has reported that triclosan increased body weight and hepatic weight with concomitant accumulation of lipid droplets in the liver. Besides, histology of liver tissues examined has been observed with severe hepatocellular atrophy and necrosis along with a reduction in mRNA and protein expression

of anti-apoptotic protein *Bcl-2* whereas the expression of pro-apoptosis protein *Bax* was significantly increased, which stated that liver injury was closely related to apoptosis in hepatocyte. In addition, the study also revealed that hepatocyte injury in zebrafish has been mediated through the up-regulation in mRNA and protein levels of the MAPK/ p53 signaling pathway (Liu *et al*., 2019).

In contrary to the above findings, a multigenerational study on Japanese medaka showed no impacts on hepatic vitellogenin, secondary sex characteristics, or sex ratio after exposure to triclosan (Mihaich *et al*., 2019). A study on bioaccumulation and toxicity of triclosan in response to climate-change-related stressors like acidification and warming has reported an enhancement of vitellogenin content, and inhibition of heat shock proteins HSP_{70}/HSC_{70} in juvenile white seabream *Diplodus sargus* (Maulvault *et al*., 2019). Exposure of triclosan to medaka *Oyzias latipes* from 8 h post-fertilization to 15 d post-fertilization has resulted in several developmental abnormalities, including enlarged yolk sac, severe edema in the pericardial region, and reduction in head trunk angle along with elevated male sex ratio, which was negatively correlated with the expression of *cyp19ala* or aromatase A and androgen receptor alpha genes. Besides, the study also reported that triclosan exposure altered the expression of genes encoding enzymes involved in DNA methylation and demethylation in primordial germ cells thereby suggested epigenetic effects on germ cells (Song *et al*., 2020). Fertilized zebrafish eggs exposed to triclosan for 120 d increased total triiodothyronine, total thyroxine, free triiodothyronine, and free thyroxine levels with an increase in the

height and nuclear area of thyroid follicular cell thereby affected neurodevelopment in the embryos (Tang *et al*., 2020).

The literature reviewed in the study portrayed that triclosan exerts endocrine disruptive effects in various fish species showing estrogenic properties. On reviewing the data collected, most of the studies were focused on the vitellogenin level and developmental abnormalities while the information on the reproductive physiology during the spawning phase of the fish remained unexplored. The data presented in this chapter provide new insights on the influence of triclosan in the spawning phase of reproduction of the fish, *Anabas testudineus*.

4. MATERIALS AND METHODS

4.1 Chemicals

Hormone assay kits were procured from Bioassay Technology Laboratory, Shanghai, China, and vitellogenin assay kit was procured from Origin Diagnostics and Research, India. All the other chemicals were purchased from the local commercial sources.

4.2 Experimental design

The experiments were carried out during the spawning stage (July - August) of the fish. Fish of both sexes were randomly selected from the stock, and introduced into the separate glass tanks (ten fish per group, in replicates). The experimental design was explained in the following Table:

Group	**Fish of Spawning stage (July-August) n = 10/ group, in replicates**		**Treatment duration**
Control	No toxicant (only tap water)		4, 7, 30, 60 d
Vehicle	Solvent - dimethylsulfoxide (DMSO; 0.001 % v/ v)		4, 7, 30, 60 d
Treatment (Triclosan)	0.009 µg L^{-1}	Environmentally relevant concentration (Ramaswamy *et al*., 2011)	4, 7, 30, 60 d
	9 µg L^{-1}	Environmentally relevant concentration (Nag *et al*., 2018)	4, 7, 30, 60 d
	176.7µg L^{-1}	Sublethal concentration (Priyatha and Chitra, 2018)	4, 7, 30, 60 d

4.3 Sample collection

At the end of every treatment period, the weight of the fish, mucus deposition, weights of gonads and liver were recorded immediately to evaluate the absolute and relative weights of the tissues. The reproductive indices such as fecundity of females, and sperm motility, viability, and counts were measured. Blood samples of both male and female fish collected from the caudal vein were kept undisturbed at room temperature for 20-30 min in a vial containing PBS heparin as an anticoagulant to obtain plasma for vitellogenin analysis. However, the serum samples were obtained by centrifuging the blood at 2000-2500 rpm for 15 min and stored at −80 °C until hormone analyses were performed. Gonads and liver tissues were then homogenized, centrifuged at 800 *g* for 15 min at 4 °C, and the supernatants obtained were used for the biochemical analysis, including vitellogenin. A portion of the gonadal tissues was preserved in 10% buffered formalin for histological analysis.

4.3.1 Fish fecundity

The fecundity of fish was estimated by the gravimetric method from the ovary of the spawning stage. The ovary was preserved in modified Gilson's fluid for 48 h to liberate the eggs completely. Then the eggs were washed thoroughly, spread on the blotting paper to air dry, and the sub-samples are counted by the given equation.

F = nG/ g, where F = fecundity, n = number of eggs in the subsample, G = total weight of the ovaries, g = weight of the subsample in the same units (Grimes and Huntsman, 1980).

4.3.2 Sperm indices

Male fish were gently held without stress, and the catheters were inserted into the urogenital tract for the collection of uncontaminated milt. The collected sperm were transferred to a small Petri plate and processed for assessing sperm motility, sperm count (Caille *et al*., (2006, and sperm viability (Eliasson, 1977; Wyrobek *et al*., .(1983

4.3.3 Vitellogenin and total protein in blood plasma, liver, and gonads

The level of vitellogenin was measured in the blood plasma, and gonadal and liver tissues using enzyme-linked immunosorbent assay (ELISA) kits, (Catalogue Number: O19086, Origin Diagnostics and Research, India), following the manufacturer's instructions. Briefly, prepared antigen standards and samples were added to 96-well plates pre-coated with primary antibodies. Microplates were incubated at 37 °C for 60 min after the addition of biotin conjugate and enzyme conjugate reagents. Then the plates were rinsed 5 times with wash solution, and the absorbance was read at 450 nm within 15 min of chromogenic reaction. Total soluble protein was also estimated as per the method of Lowry *et al*. (1951) with bovine serum albumin as the standard.

4.3.4 Serum hormones

The estimation of the levels of serum hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH),

thyroid-stimulating hormone (TSH), testosterone (T), and estradiol (E2) was performed using ELISA kits, strictly according to the manufacturer's instructions.

4.3.5 Gonadal steroidogenic enzymes

The activities of gonadal steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were estimated (Bergmeyer, 1974). The assay mixture of 3β-HSD contained pyrophosphate buffer (100 mM), NAD (0.5 mM), and dehydroisoandrosterone (0.1 mM) was read at 340 nm by the immediate addition of sample at 30 s intervals for 5 min in a spectrophotometer against the blank. The units are expressed in μmol of NAD reduced/ min/ mg protein.

The assay mixture of 17β-HSD contained pyrophosphate buffer (100 mM), NADPH (0.5 mM), and 1,4-androstenedine-3,17-dione (0.8 mM) was read at 340 nm immediately after the addition of sample at 30 s interval for 5 min in a spectrophotometer against the blank. The enzyme activity was expressed as μmol of NADP formed/ min/ mg protein.

4.3.6 Histological analysis

Gonads of male and female fish, fixed in 10% buffered formalin for 24-48 h were used for histological analysis. The tissues were dehydrated in ascending grades of alcohol and cleared in xylene until they become translucent. The tissues were embedded in molten paraffin wax for an hour for the complete impregnation to make the

tissue blocks. Sections of 4 to 6 μm thickness were prepared using a rotary microtome, and then the slides obtained were double-stained with hematoxylin and eosin, and finally mounted in DPX (Roberts and Smail, 2001). Slides of gonadal tissues were examined under the microscope and photographed.

4.4. Statistical analyses

In the present study, all biochemical analyses were performed in triplicates to minimize the statistical errors. Data analyses were performed using SPSS software, version 21.0. All data were expressed as mean ± standard deviation (SD) for ten animals per group, in replicates. One-way ANOVA followed by Duncan's Multiple Range post-hoc was used to set the significance among the control and the treatment groups. Normal distribution and homogeneity of variance were checked before conducting One-way ANOVA, and the values considered as significant at $P < 0.05$ were denoted as asterisks (*) in the Figures.

5. RESULTS

5.1 Effect of triclosan on the body weight and mucus deposition in the fish, *Anabas testudineus*

Fish exposed to triclosan at three different concentrations namely 0.009, 9, and 176.7 µg L^{-1} showed a significant ($P<0.05$) decrease in the weight of the animal after 30 and 60 d while no significant changes were observed after 4 and 7 d of the treatment (Fig. 1). However, the mucus deposition increased significantly ($P<0.05$) at all concentrations after 4, 7, 30, and 60 d of triclosan exposure (Fig. 2).

5.2 Effect of triclosan on the absolute and relative weights of gonads and liver tissues

The absolute and relative weights of gonads, the ovary, and testis, showed a significant ($P<0.05$) reduction after 30 and 60 d of triclosan treatment at all concentrations when compared with the corresponding control groups (Figs. 3 and 4). In female fish, the absolute weight of liver tissues decreased significantly ($P<0.05$) while male liver tissues showed significant ($P<0.05$) increase after 30 and 60 d of all treatment groups (Fig. 5). The hepatosomatic index (HSI) in female fish showed significant ($P<0.05$) reduction only in sublethal exposure group after 30 and 60 d while HSI of male fish increased significantly ($P<0.05$) in all treatment groups at the end of 30 and 60 d when compared to the respective control groups (Fig. 6).

5.3 Effect of triclosan on the concentration of total protein in the plasma, gonads, and liver tissues

Fish exposed to triclosan showed significant ($P<0.05$) increase in the concentration of total plasma protein in a time-dependent manner in all treatment groups than the corresponding controls (Fig. 7). In ovary, triclosan exposure at 0.009 µg L^{-1} concentration for 30 and 60 d, and after 7 d onwards at 9 and 176.7 µg L^{-1} concentrations showed a significant ($P<0.05$) reduction in the total protein (Fig. 8). Triclosan exposure on testis significantly ($P<0.05$) declined the level of total protein in all treatment groups after 30 and 60 d than that of the respective control groups (Fig. 8). Similarly, a concentration- and time-dependent significant ($P<0.05$) reduction in the level of total protein was observed after triclosan exposure in the liver tissue of the fish in comparison with their control groups (Fig. 9).

5.4 Effect of triclosan on the level of vitellogenin in blood plasma, gonads, and liver tissues

In female fish, the level of vitellogenin in the blood plasma and liver tissue increased significantly ($P<0.05$) after 30 and 60 d in all concentration groups in duration- and concentration-dependent manner (Fig. 10) while the ovarian tissue showed significant ($P<0.05$) decline in the level of vitellogenin only after 30 and 60 d (Fig. 10).

In male fish, triclosan exposure significantly ($P<0.05$) increased the level of vitellogenin in blood plasma, testis, and liver tissues in a time- and concentration-dependent manner when compared with the corresponding control groups (Fig. 11).

5.5 Effect of triclosan on the levels of serum hormones

The level of serum estradiol decreased significantly ($P<0.05$) after 30 and 60 d at 0.009 and 9 µg L^{-1} concentrations while a significant ($P<0.05$) reduction after 7 d onwards was observed at 176.7 µg L^{-1} concentration in the female fish (Fig. 12). A significant ($P<0.05$) rise in the level of estradiol was observed after 30 and 60 d of triclosan exposure at all concentrations in the male fish (Fig. 12).

The level of serum testosterone in the female fish significantly ($P<0.05$) reduced only after 30 and 60 d in all exposure groups (Fig. 13). The male fish showed a significant ($P<0.05$) decrease in the level of serum testosterone after 30 and 60 d of triclosan exposure at 0.009 µg L^{-1} concentration group. However, a significant ($P<0.05$) and time-dependent reduction in testosterone level was observed after 4 d onwards in 9 and 176.7 µg L^{-1} concentration groups than the respective control samples (Fig. 13).

Triclosan treatment significantly ($P<0.05$) decreased the levels of serum FSH and LH in both sexes in a time- and concentration-dependent manner when compared with the corresponding control groups (Figs. 14 and 15). However, the level of TSH in the serum of male and female fish significantly ($P<0.05$) increased based on exposure period and concentrations (Fig. 16).

5.6 Effect of triclosan on the activities of gonadal steroidogenic enzymes

The activities of gonadal steroidogenic enzymes namely 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid

dehydrogenase (17β-HSD) decreased significantly ($P<0.05$) in both testis and ovary at all tested concentrations in a time-dependent manner when compared with the respective control groups (Figs. 17 and 18).

5.7 Effect of triclosan on fecundity in female fish

The rate of fecundity measured by evaluating the number of eggs was found to decreased significantly ($P<0.05$) after 30 and 60 d of environmentally relevant concentration groups while the decline in fecundity was noted immediately after 7 d onwards in the sublethal triclosan exposure group (Fig. 19).

5.8 Effect of triclosan on sperm parameters

The sperm parameters such as sperm motility, sperm count, and sperm viability decreased significantly ($P<0.05$) with respect to the duration and concentration of triclosan exposure than the respective control groups (Figs. 20-22). The percentage of viable sperm were tested using trypan blue stain, which showed no stain absorption in the control groups as evidenced by the white, bright sperm while the triclosan-exposed groups at all concentrations and durations were observed with dead sperm in blue color as a result of the stain absorption, along with some morphological abnormalities like the absence of tail and enlarged sperm head. Representative viable sperm from the control groups and non-viable sperm from the treatment groups were shown in Fig. 23.

5.9 Effect of triclosan on the histology of ovary

Anabas testudineus was exposed to triclosan during the spawning phase, i.e., July and August. The ovaries of control and vehicle-control groups showed mature oocytes with centrally located large oil vacuole, and the loosely arranged spherical eggs (Figs. 24A and 24B). Triclosan exposure at 0.009 µg L^{-1} concentration for 4, 7, 30 and 60 d showed significant histological lesions in the ovary of the fish (Figs. 24C-24F). Fish exposed to triclosan for 4 d were observed with vitellogenic oocytes than the expected mature oocytes along with membrane damage and empty ovarian follicles (Fig. 24C). The intensity of oocyte membrane damage increased with the period of exposure, and at sublethal concentration, several empty follicles were found (Figs. 24D-24F).

Triclosan exposed at 9 µg L^{-1} concentration for 4, 7, 30, and 60 d, respectively showed only vitellogenic oocytes (Figs. 24G-24J) with similar lesions as observed at 0.009 µg L^{-1} concentration but the severity of tissue damage was increased. Sublethal exposure of triclosan i.e., 176.7 µg L^{-1} for 4, 7, 30, and 60 d, respectively were shown in Figs. 24K-24N, in which vitellogenic oocytes with membrane damage, empty and atretic follicles followed by the development of anucleated early-stage oocytes were prominent (Figs. 24K-24N). Some of the more prominent pathologies such as the formation of anucleated perinucleolus oocyte, degenerated oocytes with membrane damage as well as atretic and empty follicles observed after exposure to different concentrations of triclosan were represented in Fig. 24a.

5.10 Effect of triclosan on the histology of testis

Histology of testis in the spawning phase of *Anabas testudineus* showed severe histological abnormalities after the triclosan exposure than the corresponding control groups. The histology of control testis was normal with different stages of spermatogenesis arranged within the compact seminiferous tubules (Figs. 25A and 25B). Triclosan exposed at 0.009 µg L^{-1} for 4, 7, 30, and 60 d, respectively showed loss of spermatozoa with an increase in the duration of exposure. Mass-like lesions named spermatocyst were noted that distort the testicular architecture after triclosan exposure (Figs. 25C-25E). Besides, the formation of vacuolization and thickened epithelium of seminiferous tubules were also observed in the treatment groups (Figs. 25E and 25F). In the 9 µg L^{-1} concentration group, the prominent lesions were characterized by the formation of vacuolization, thickened epithelium of seminiferous tubules, and the complete loss or reduction in the number of spermatozoa (Figs. 25G-25J). The intensity of testicular damage increased at 176.7 µg L^{-1} concentration indicated by the loss of spermatozoa, vacuolization, and thickened interstitium (Figs. 25K-25N). Triclosan exposure at 176.7 µg L^{-1} concentration developed testis-ova with distinct zones of ovarian and testicular tissues within the same gonadal tissue of the fish *Anabas testudineus* (Fig. 25a).

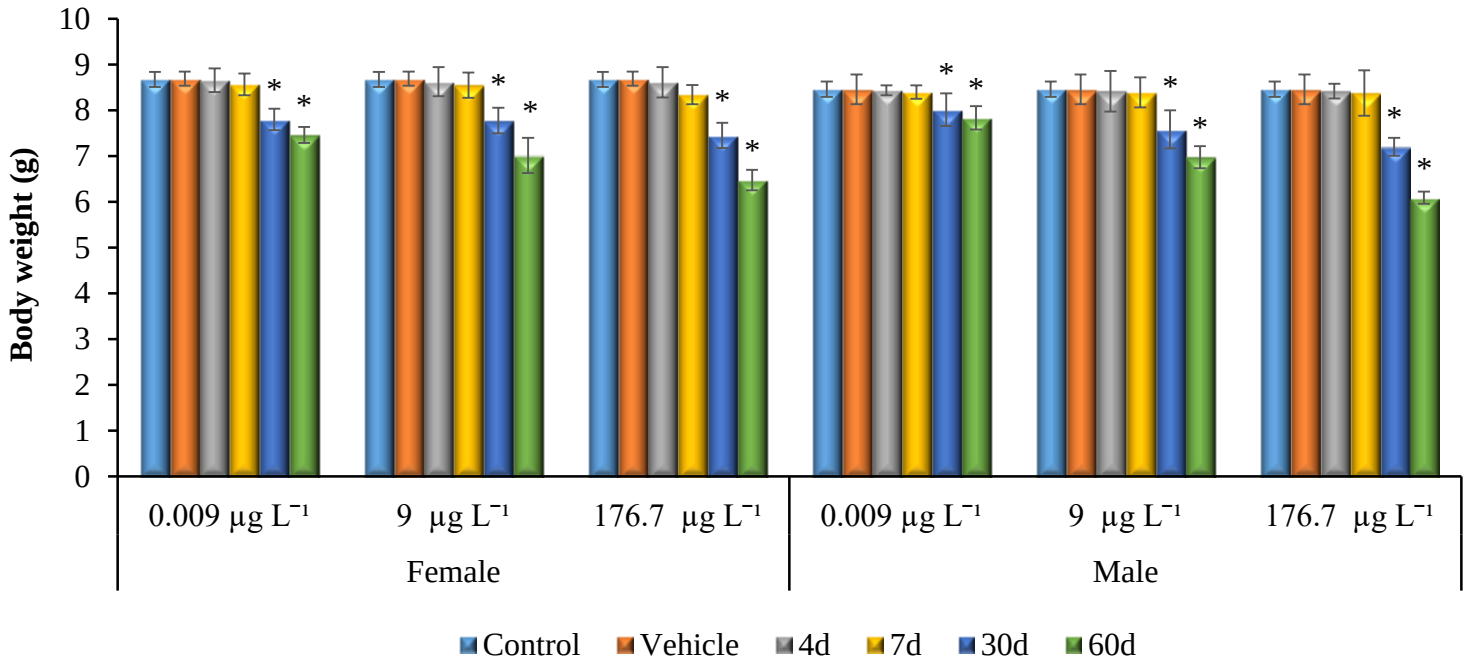


Fig. 1 **Effect of triclosan on the body weight of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

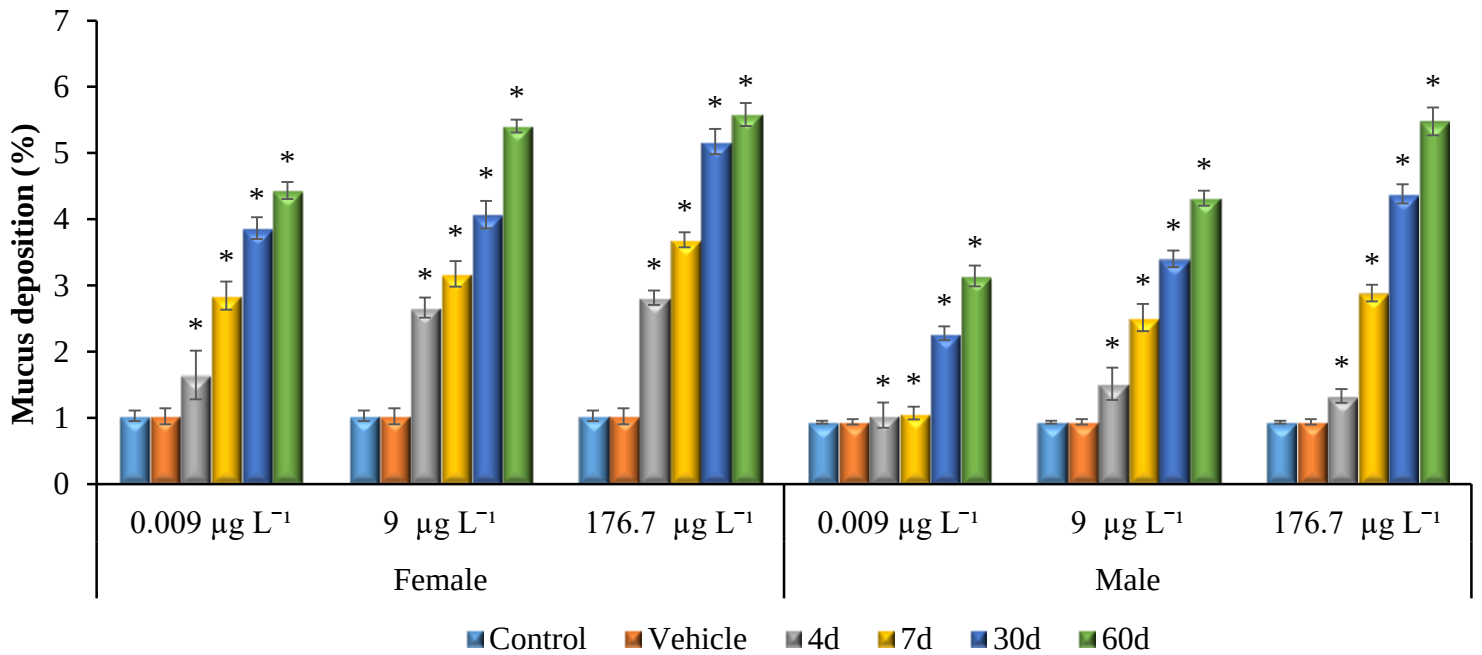
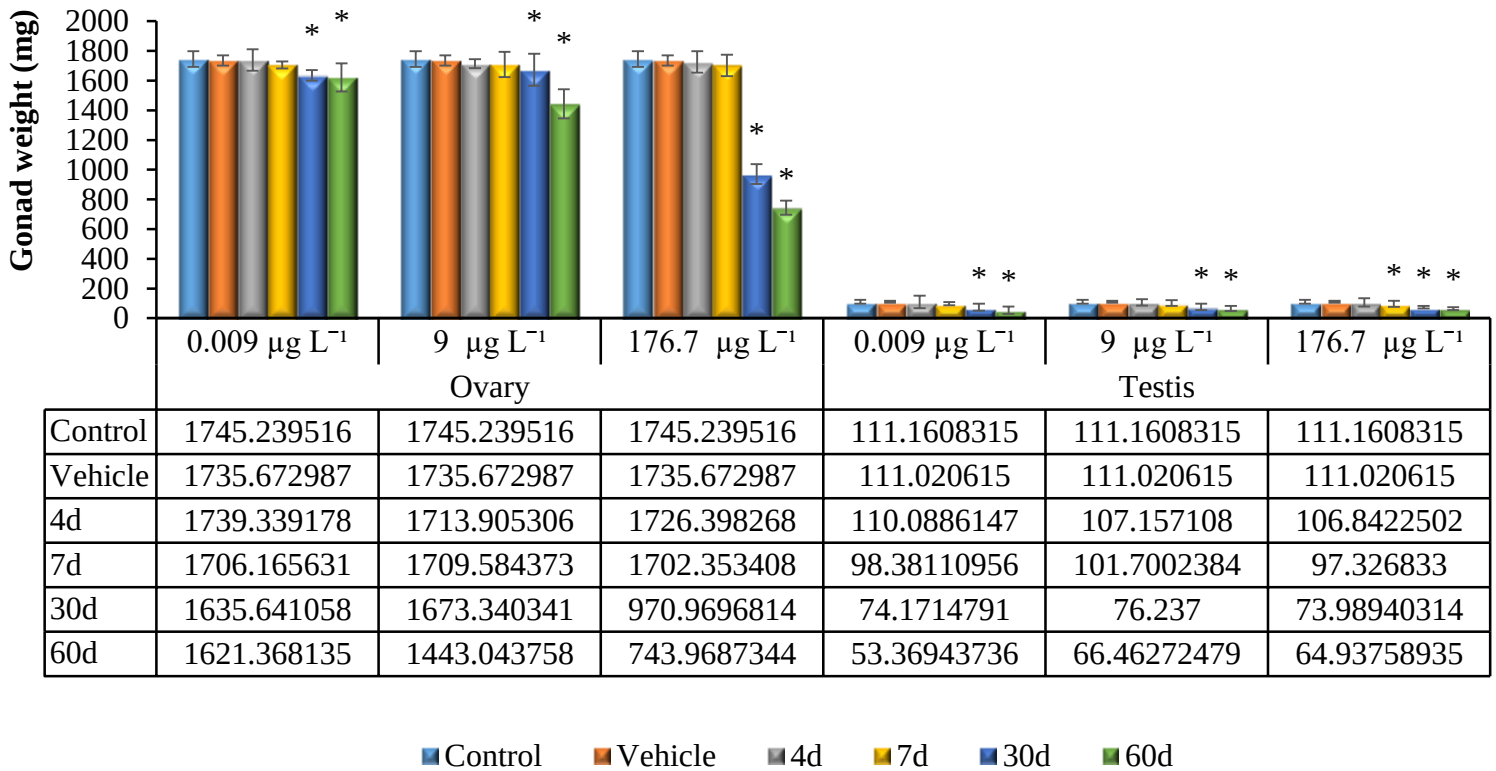
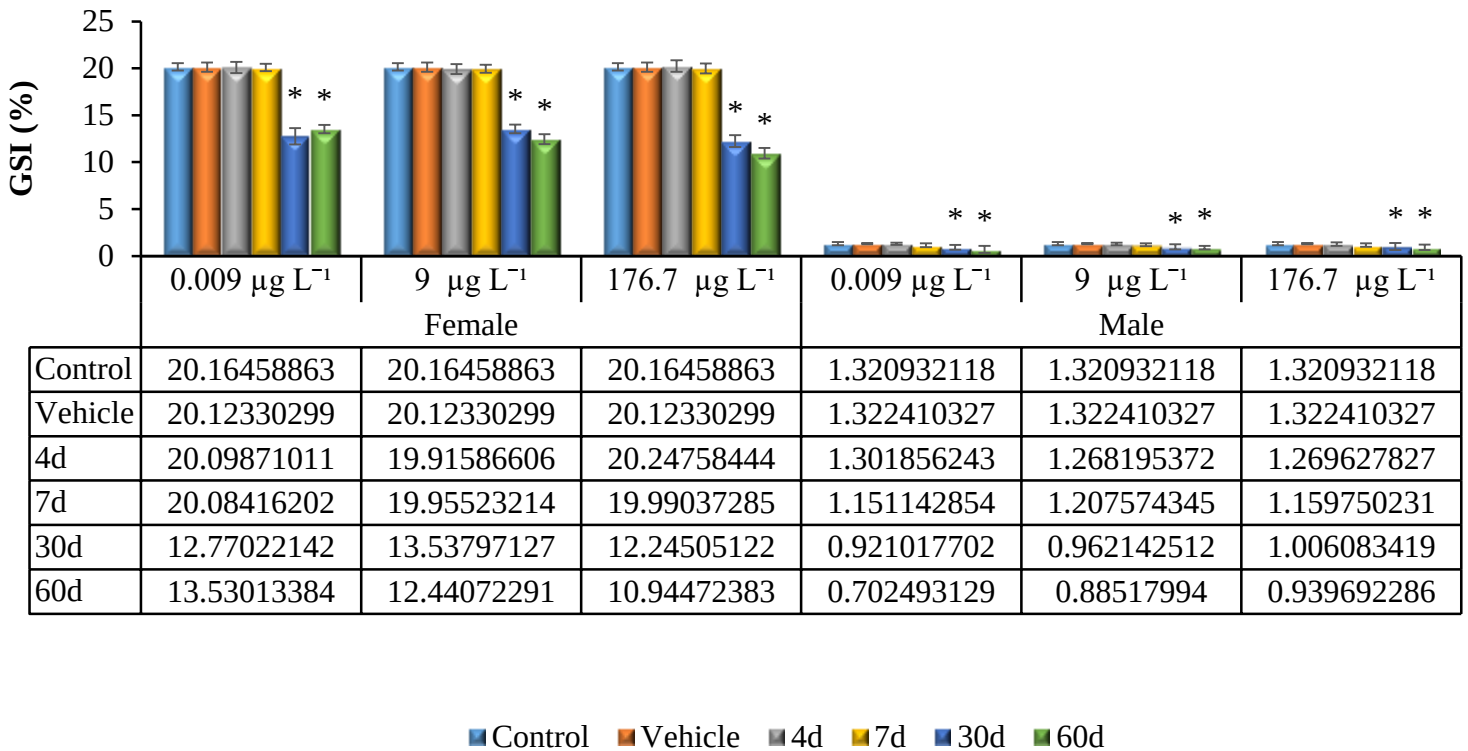


Fig. 2 **Effect of triclosan on the mucus deposition in the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**



	0.009 µg L⁻¹	9 µg L⁻¹	176.7 µg L⁻¹	0.009 µg L⁻¹	9 µg L⁻¹	176.7 µg L⁻¹
	Ovary			Testis		
Control	1745.239516	1745.239516	1745.239516	111.1608315	111.1608315	111.1608315
Vehicle	1735.672987	1735.672987	1735.672987	111.020615	111.020615	111.020615
4d	1739.339178	1713.905306	1726.398268	110.0886147	107.157108	106.8422502
7d	1706.165631	1709.584373	1702.353408	98.38110956	101.7002384	97.326833
30d	1635.641058	1673.340341	970.9696814	74.1714791	76.237	73.98940314
60d	1621.368135	1443.043758	743.9687344	53.36943736	66.46272479	64.93758935

Fig. 3 **Effect of triclosan on the weights of gonads in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**



	Female			Male		
	0.009 µg L⁻¹	9 µg L⁻¹	176.7 µg L⁻¹	0.009 µg L⁻¹	9 µg L⁻¹	176.7 µg L⁻¹
Control	20.16458863	20.16458863	20.16458863	1.320932118	1.320932118	1.320932118
Vehicle	20.12330299	20.12330299	20.12330299	1.322410327	1.322410327	1.322410327
4d	20.09871011	19.91586606	20.24758444	1.301856243	1.268195372	1.269627827
7d	20.08416202	19.95523214	19.99037285	1.151142854	1.207574345	1.159750231
30d	12.77022142	13.53797127	12.24505122	0.921017702	0.962142512	1.006083419
60d	13.53013384	12.44072291	10.94472383	0.702493129	0.88517994	0.939692286

Fig. 4 **Effect of triclosan on the gonadosomatic index (GSI) of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

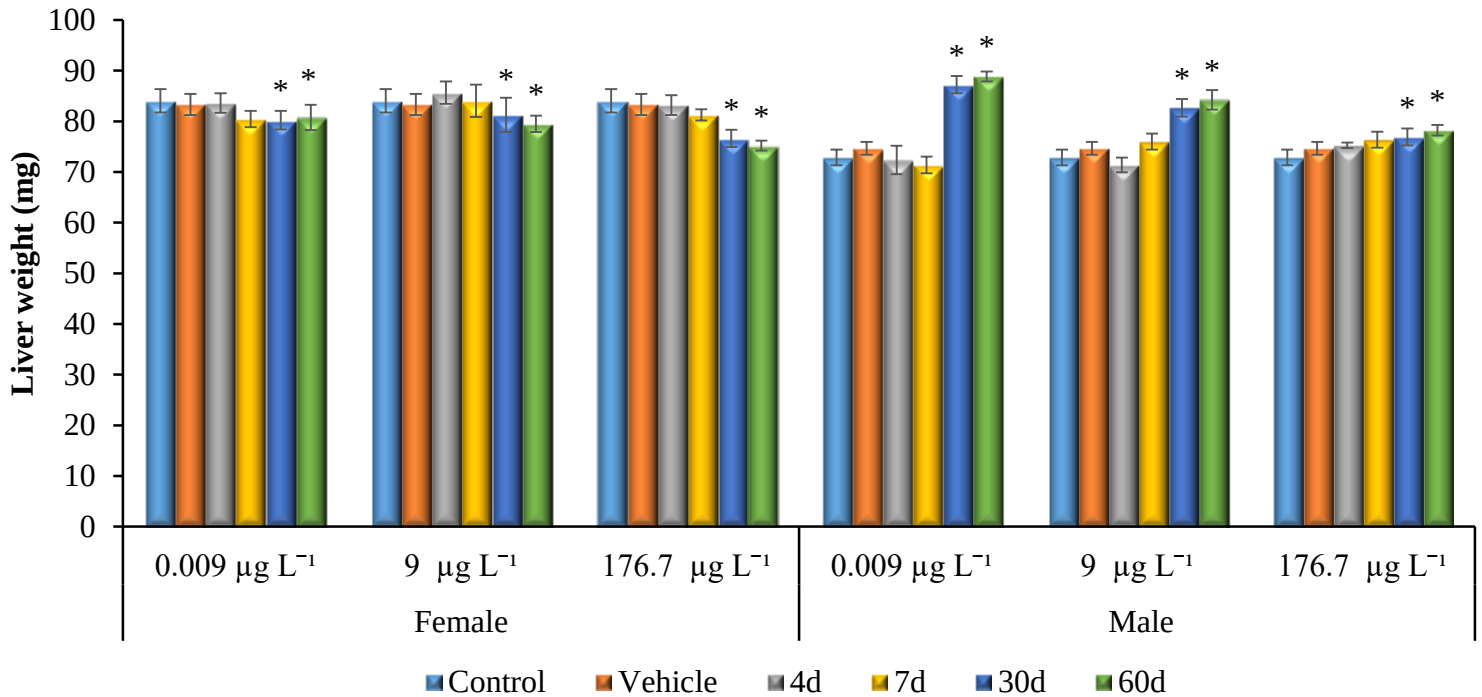


Fig. 5 **Effect of triclosan on the weight of liver tissue in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

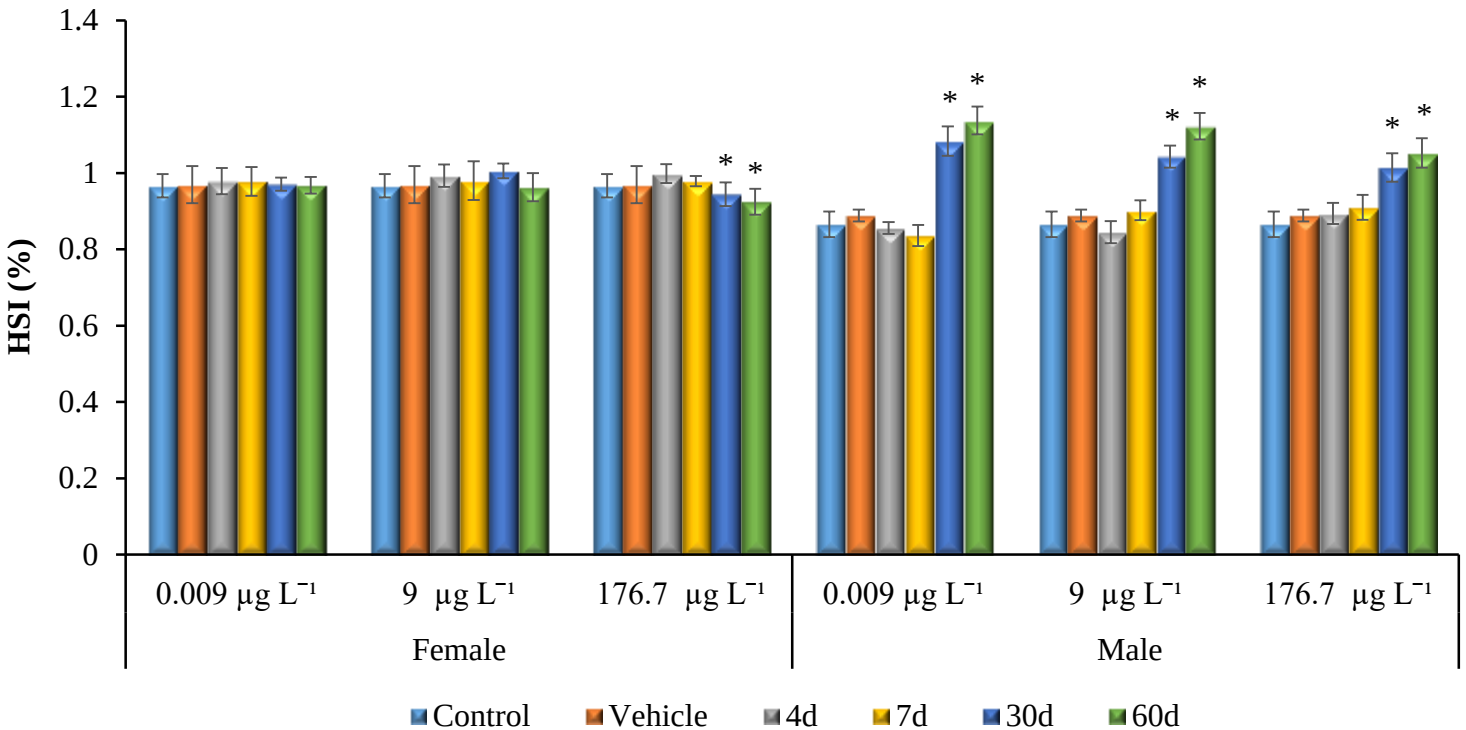


Fig. 6 **Effect of triclosan on the hepatosomatic index (HSI) of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

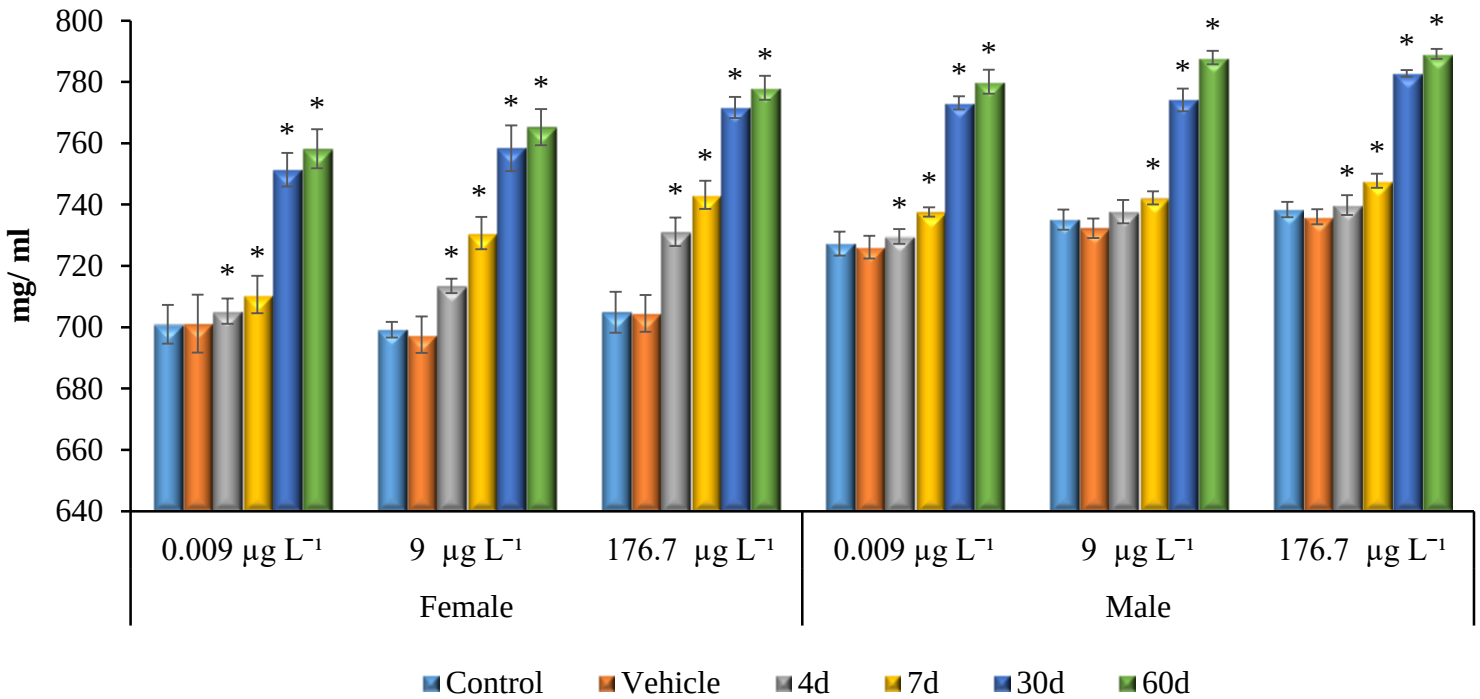


Fig. 7 **Effect of triclosan on the level of total plasma protein in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

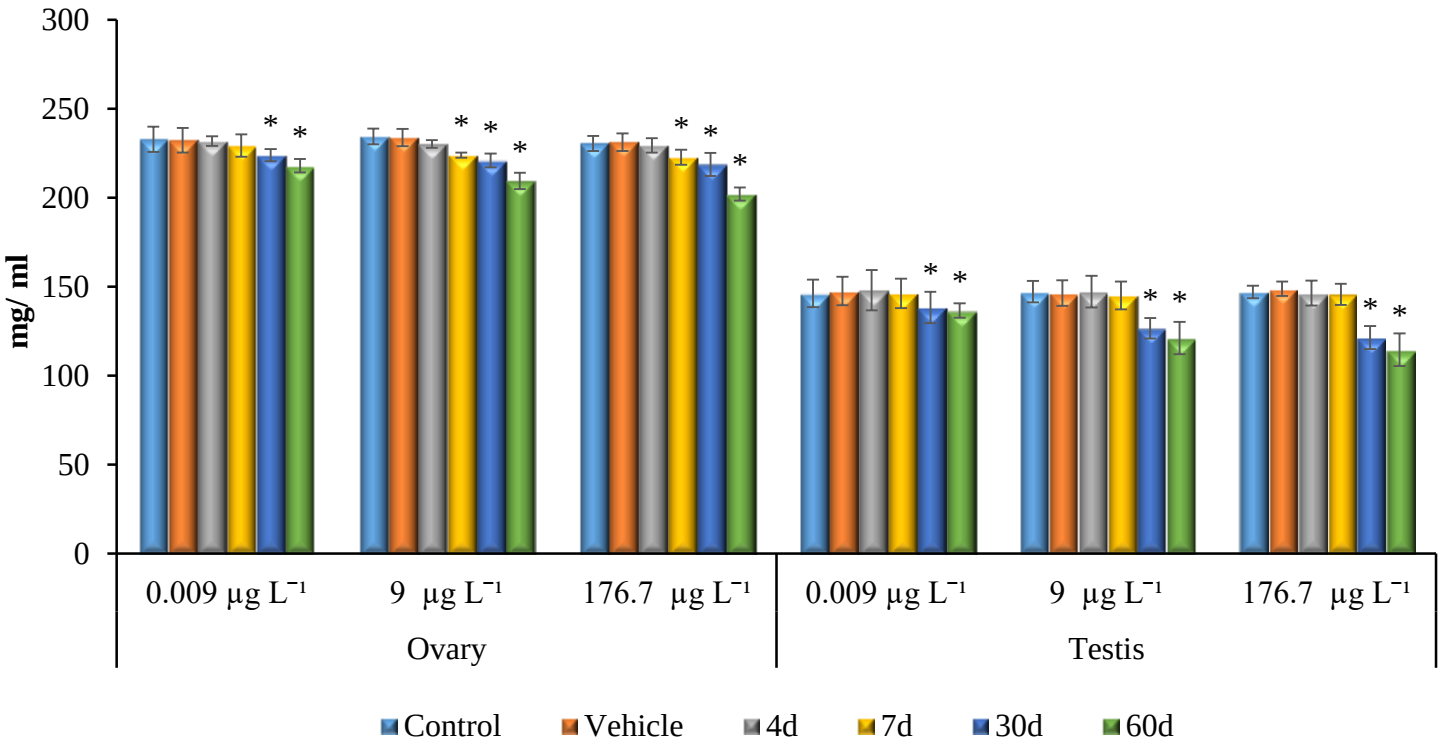


Fig. 8 **Effect of triclosan on the level of total protein in the gonads of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

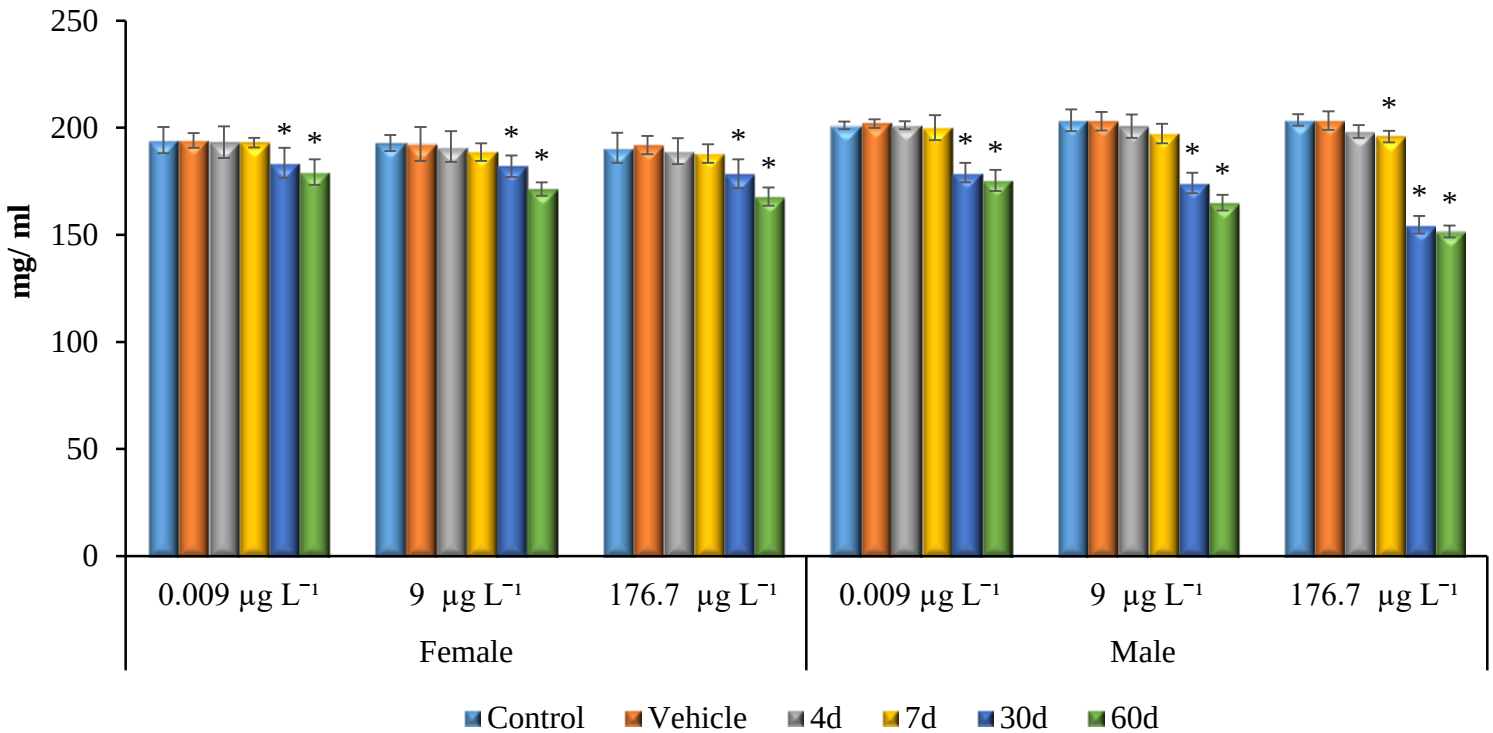


Fig. 9 **Effect of triclosan on the level of total protein in the liver tissue of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

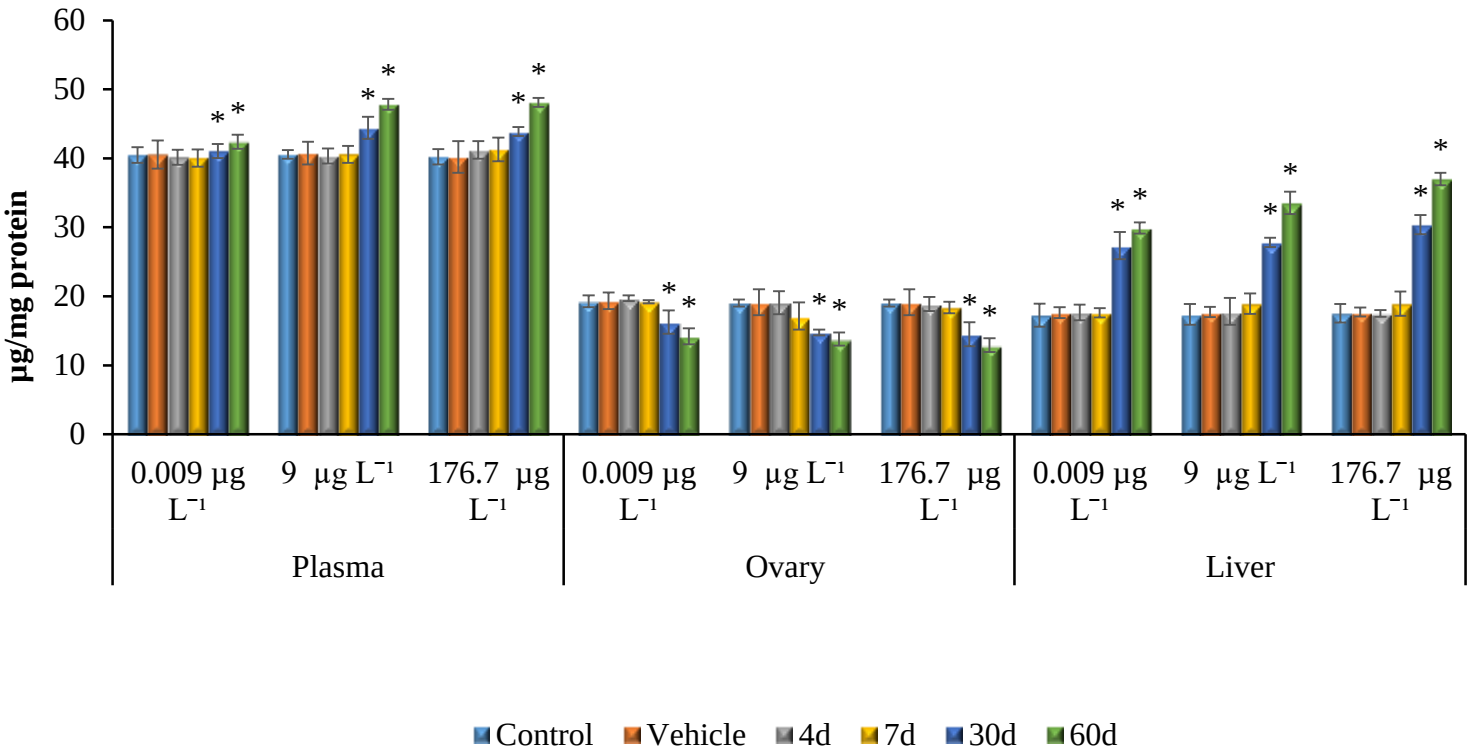


Fig. 10 **Effect of triclosan on the level of vitellogenin in the female fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

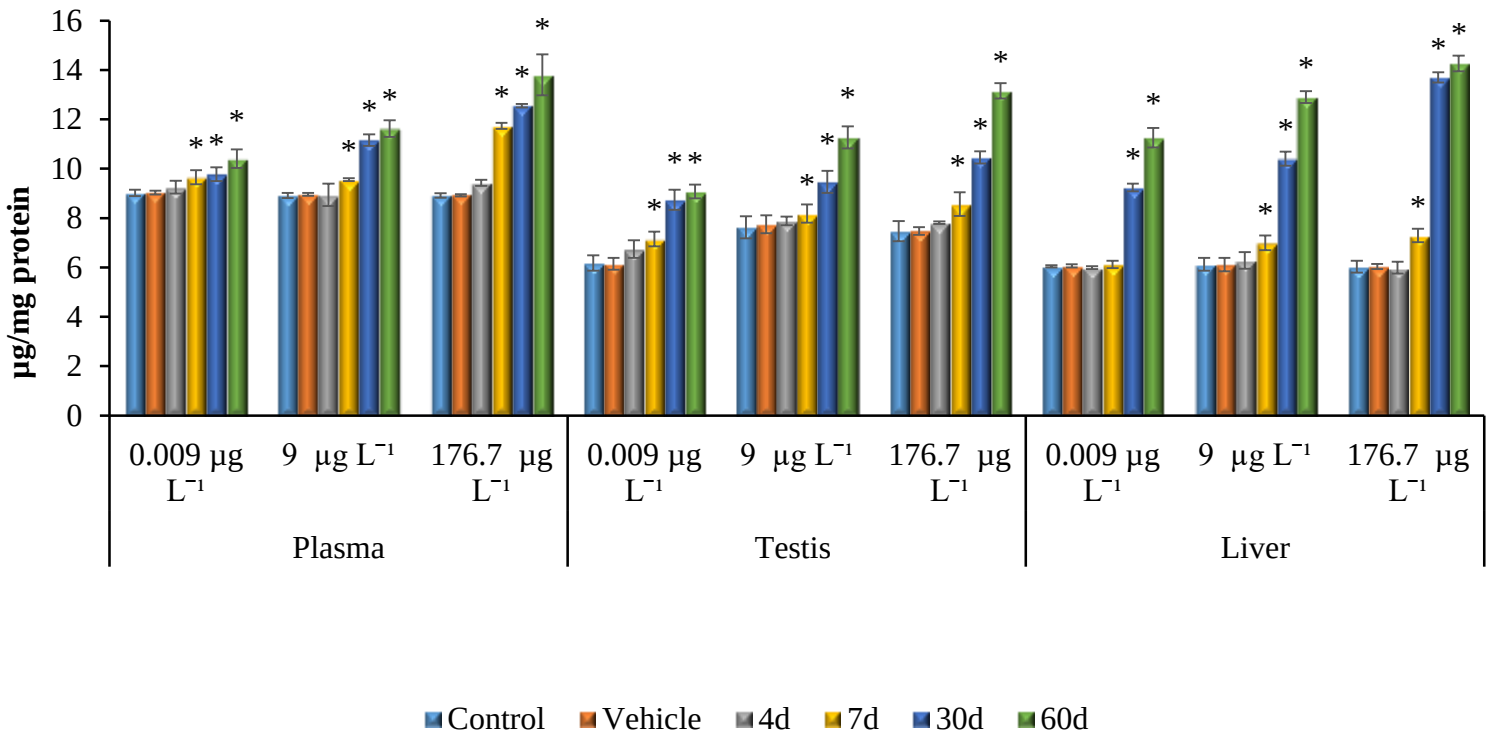


Fig. 11 **Effect of triclosan on the level of vitellogenin in the male fish *Anabas testudineus* (Mean ± SD; n = 10/ group, , in replicates; Significant at *P<0.05 against the control groups)**

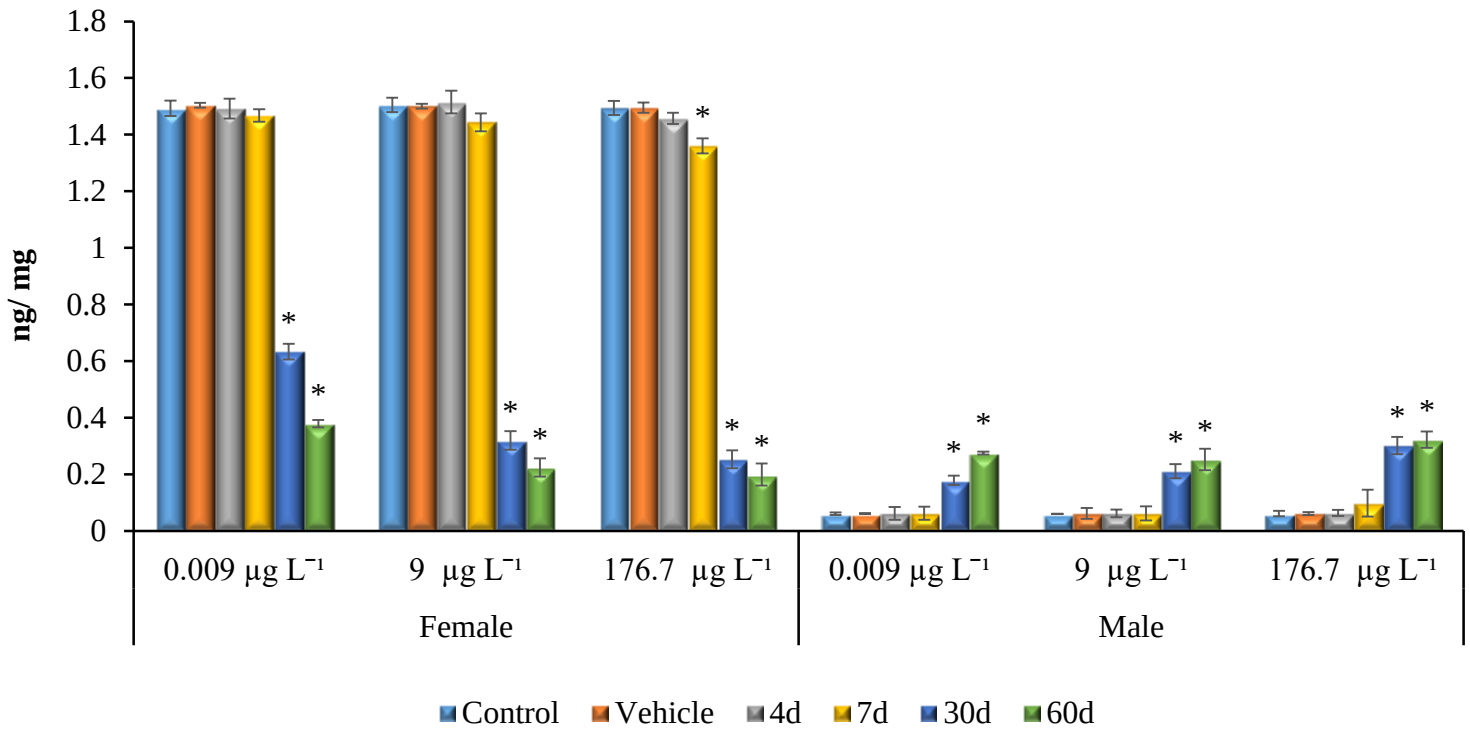


Fig. 12 **Effect of triclosan on the level of serum estradiol in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

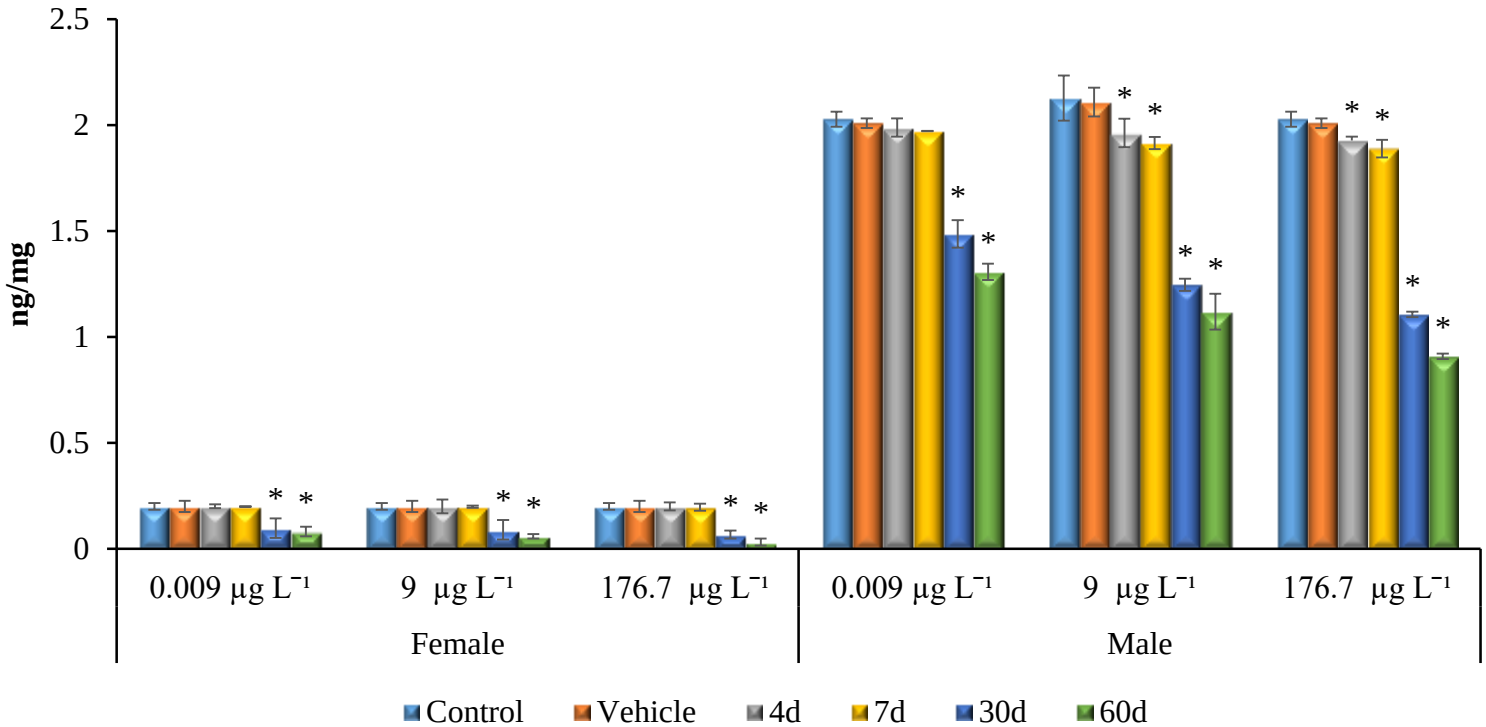


Fig. 13 **Effect of triclosan on the level of serum testosterone in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

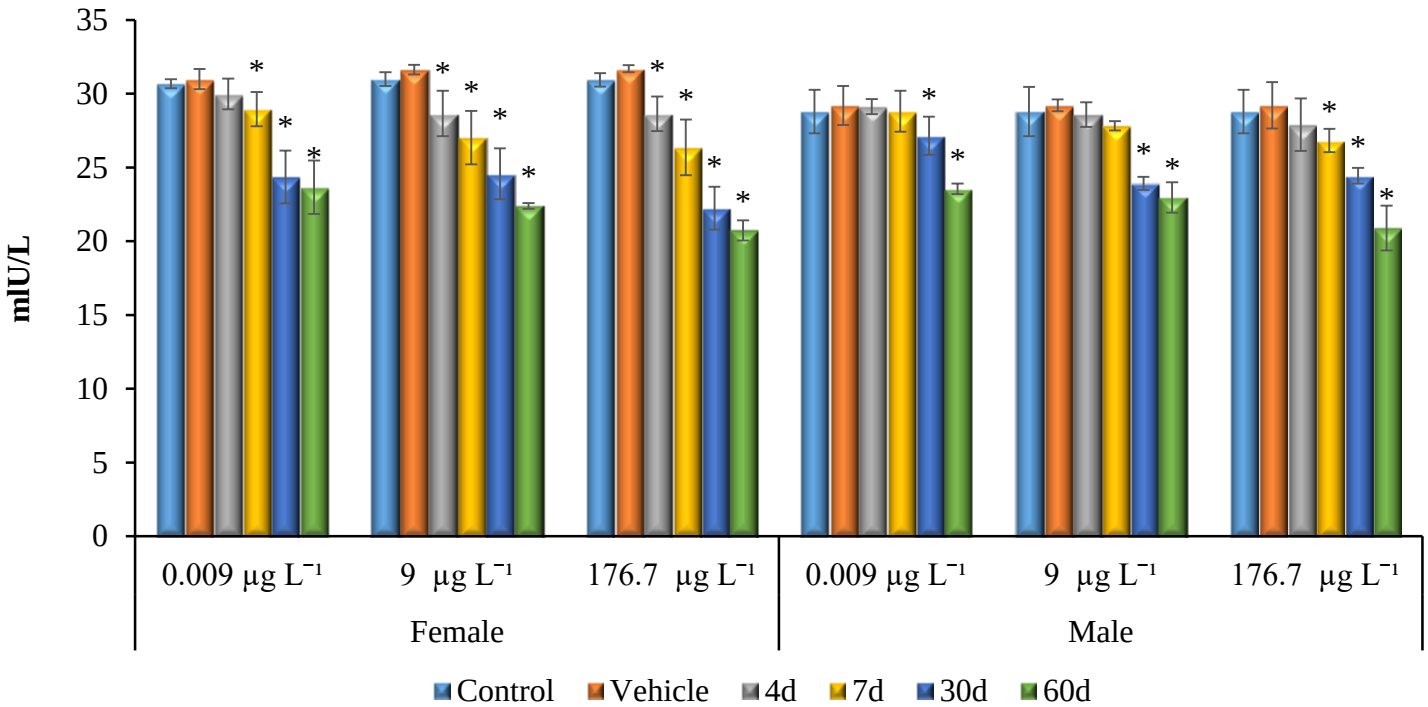


Fig. 14 **Effect of triclosan on the level of follicle stimulating hormone (FSH) in the serum of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

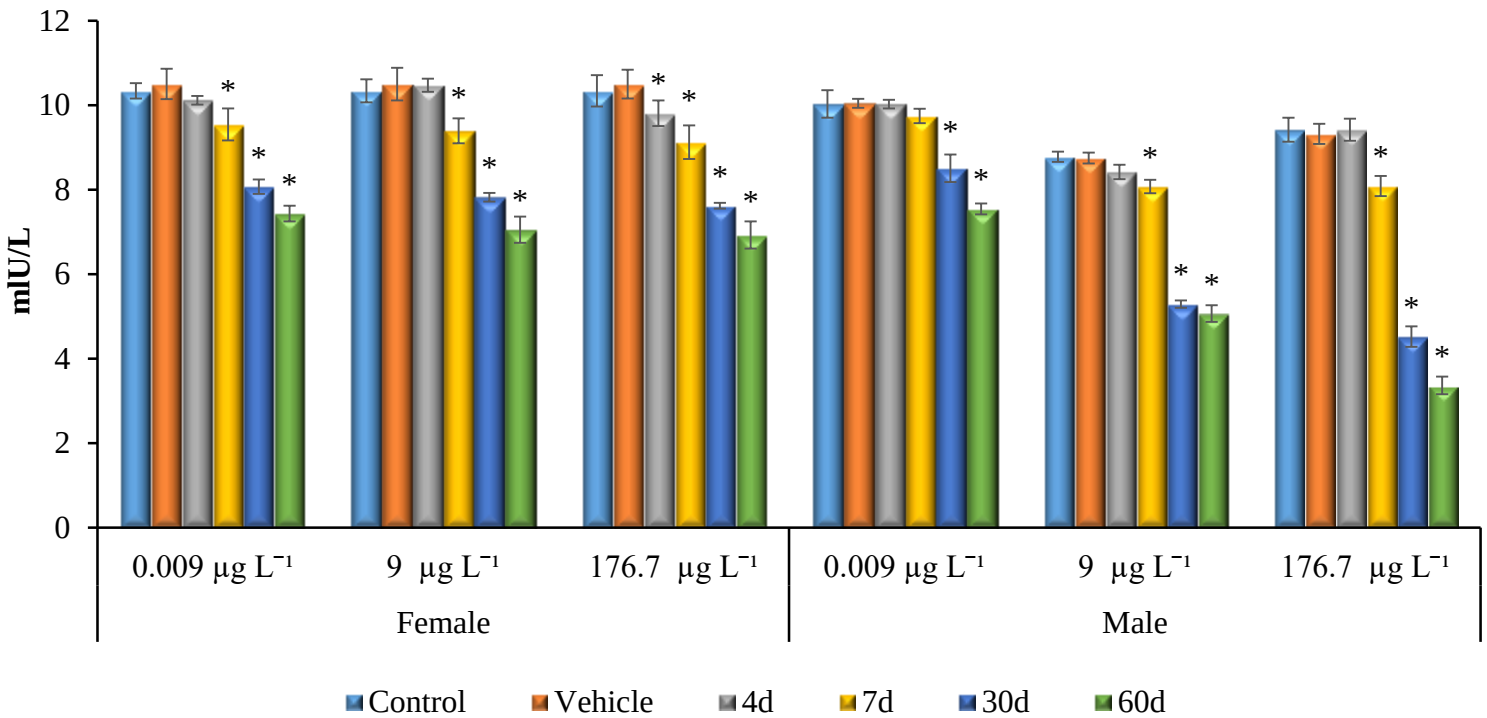


Fig. 15 **Effect of triclosan on the level of luteinizing hormone (LH) in the serum of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

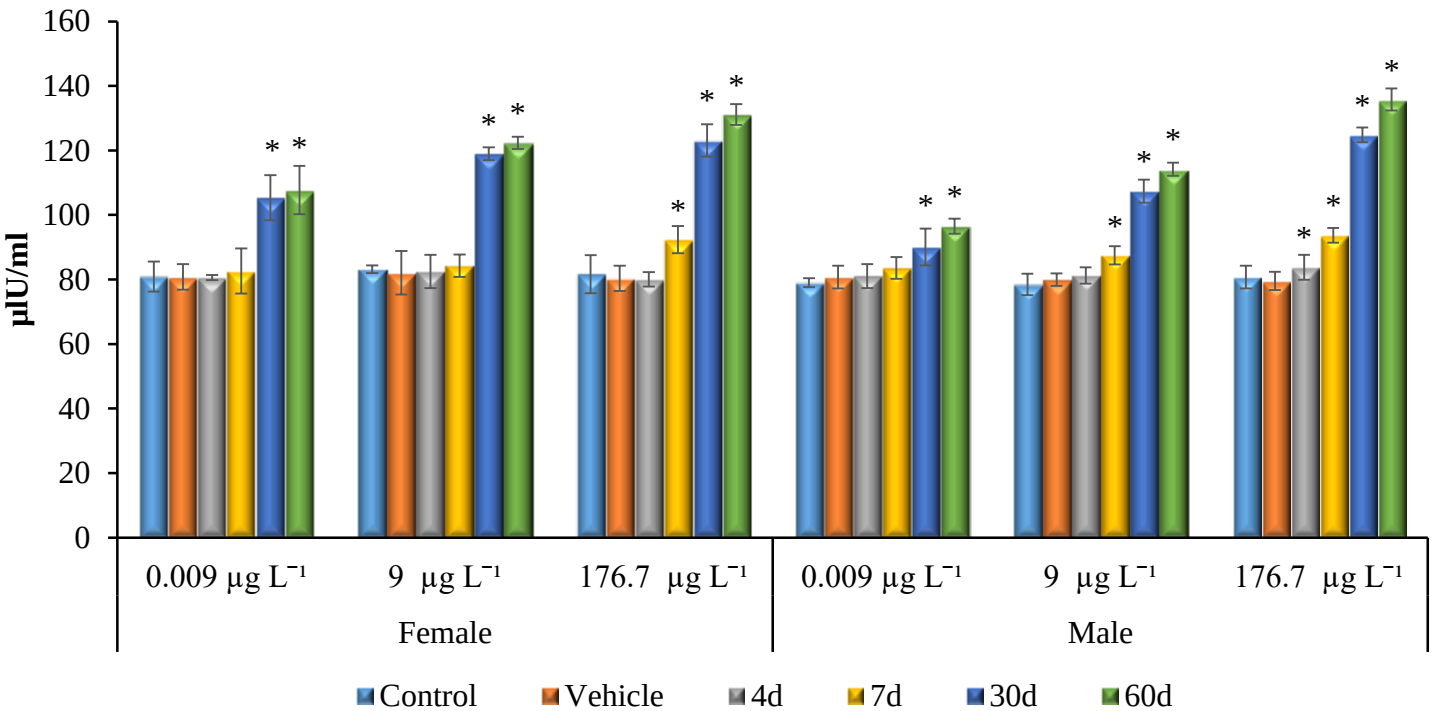


Fig. 16 **Effect of triclosan on the level of thyroid stimulating hormone (TSH) in the serum of the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

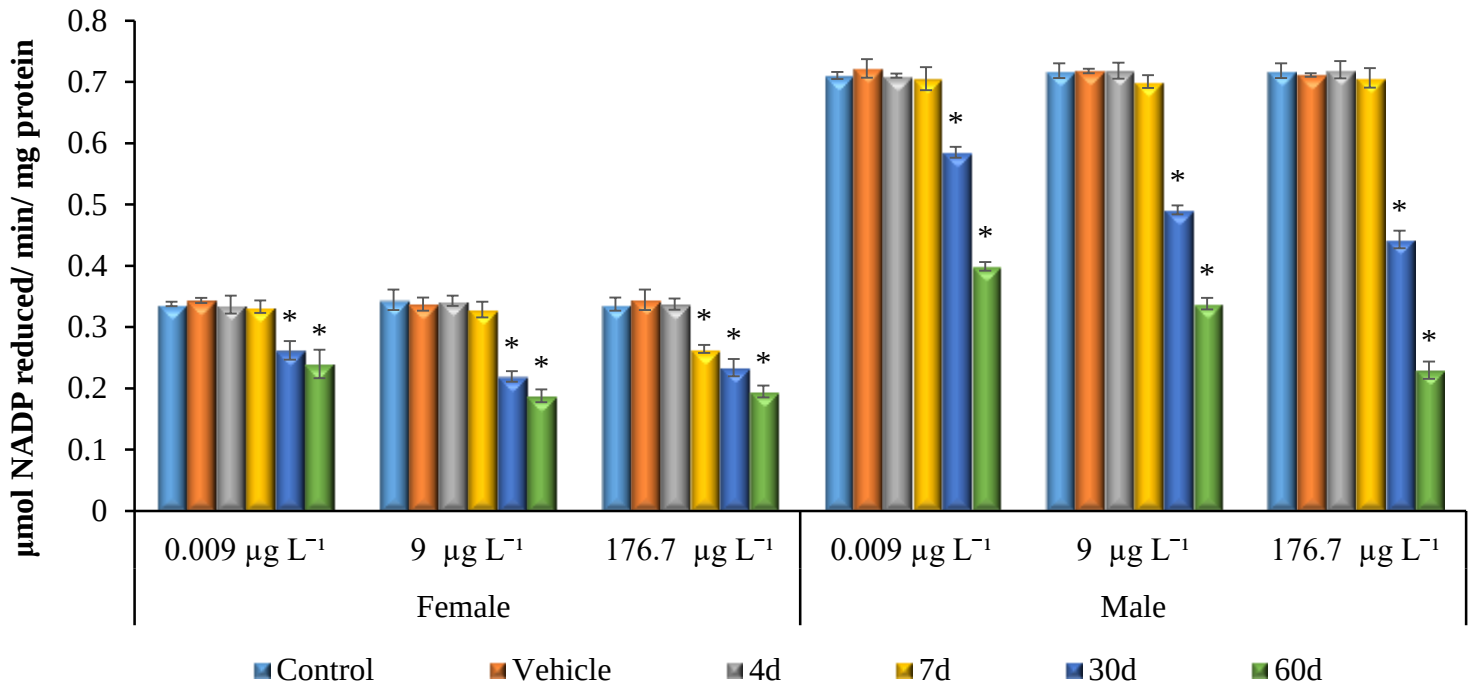


Fig. 17 **Effect of triclosan on the activity of 3β-hydroxysteroid dehydrogenase in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

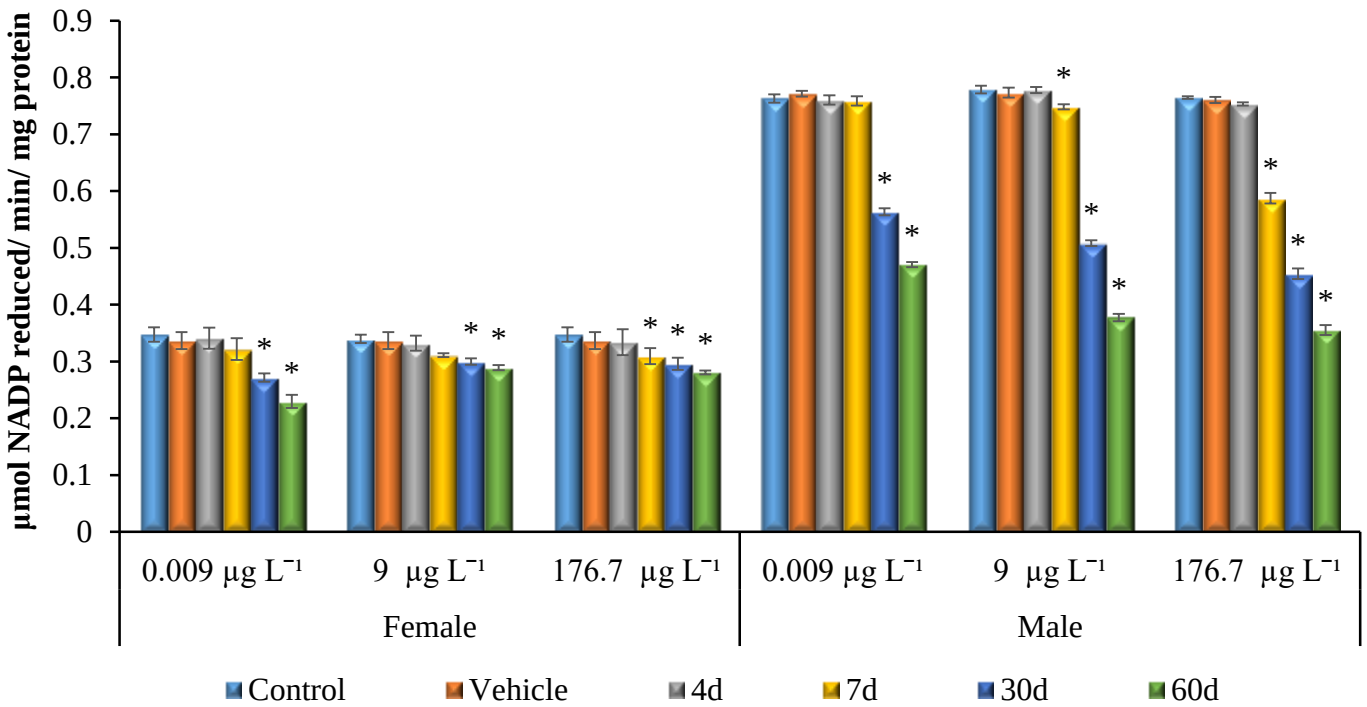


Fig. 18 **Effect of triclosan on the activity of 17β-hydroxysteroid dehydrogenase in the fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

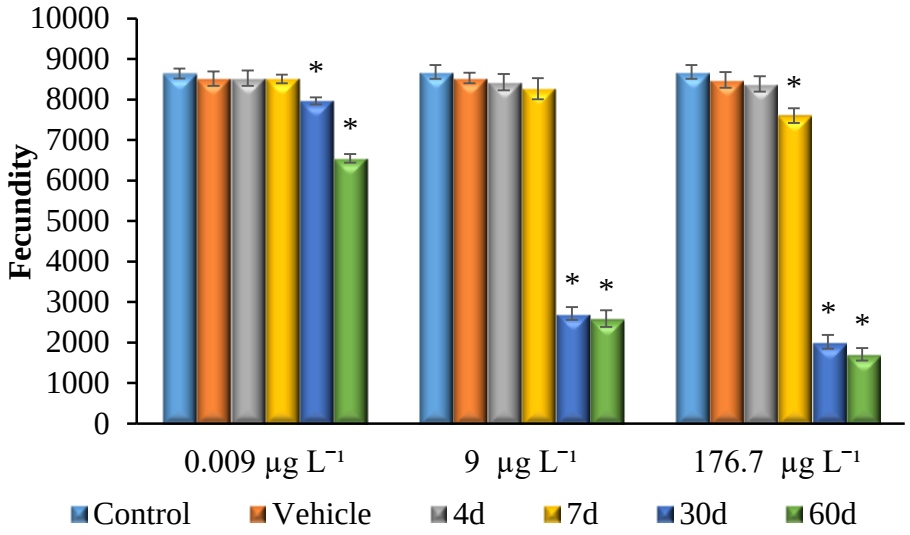


Fig. 19 **Effect of triclosan on the rate of fecundity in the female fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

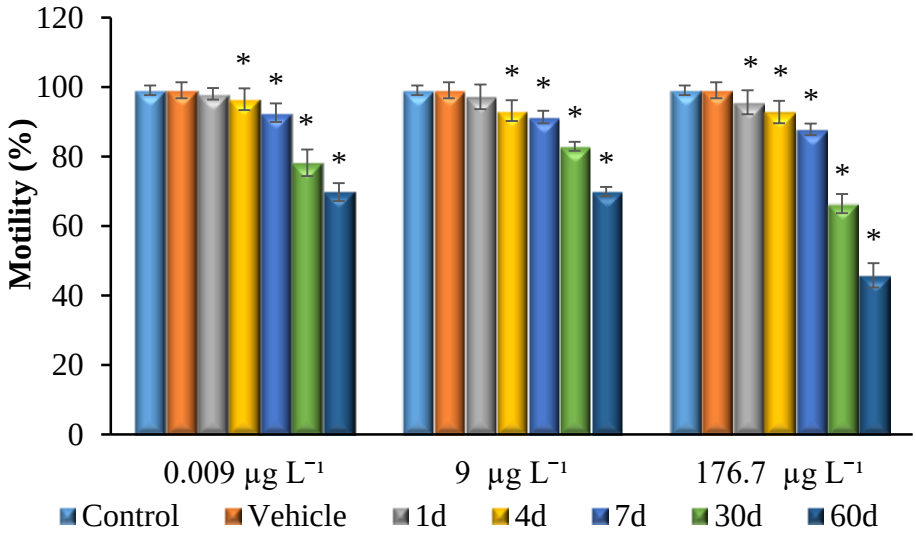


Fig. 20 **Effect of triclosan on the sperm motility in the male fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

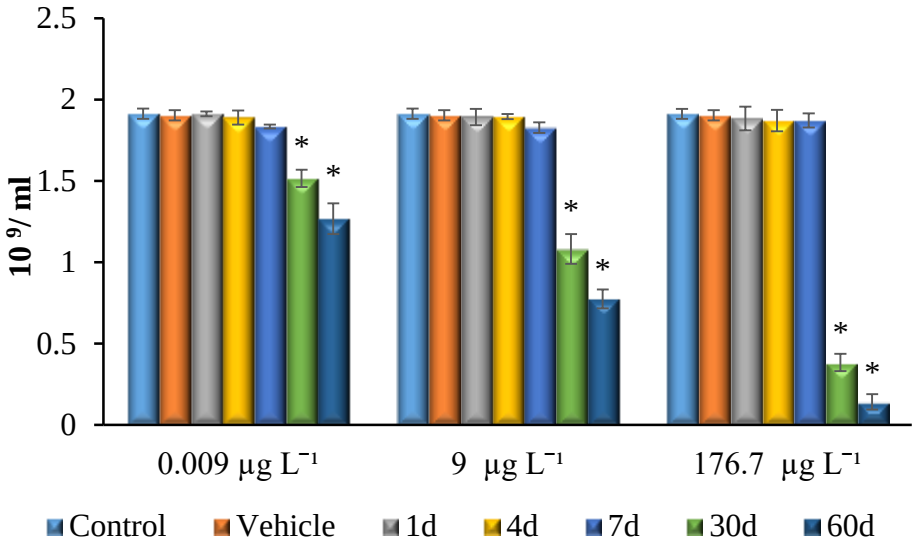


Fig. 21 **Effect of triclosan on the sperm count in the male fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

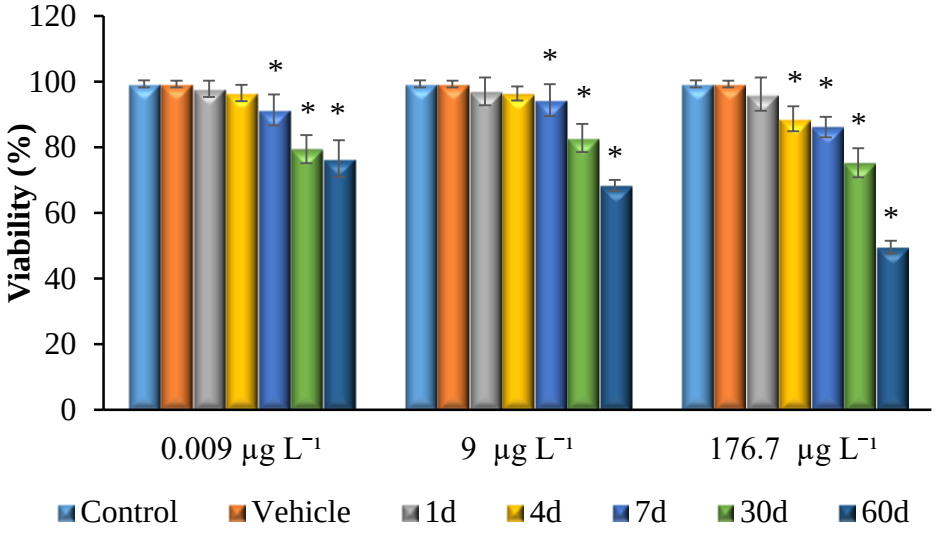


Fig. 22 **Effect of triclosan on the sperm viability in the male fish *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)**

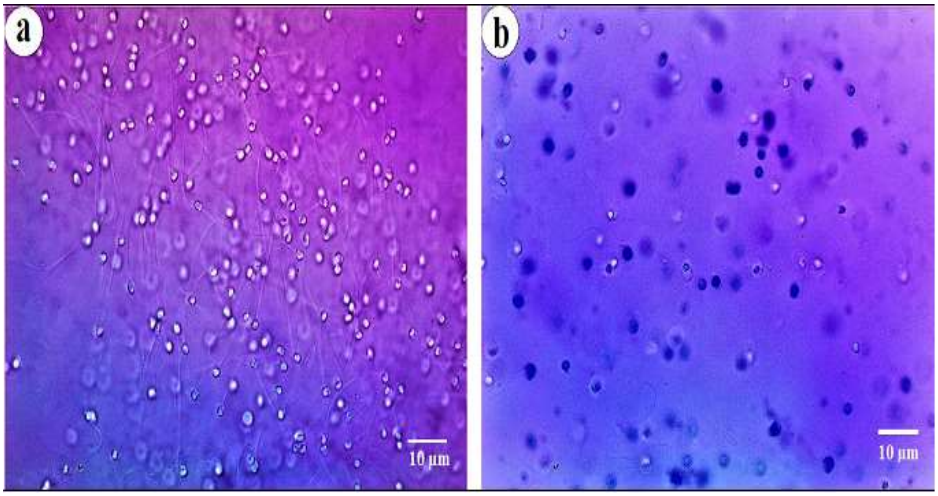


Fig. 23 **Representative image on the sperm viability of the fish *Anabas testudineus* (a) Viable sperm (control groups); (b) Non-viable sperm (Triclosan-exposed groups)**

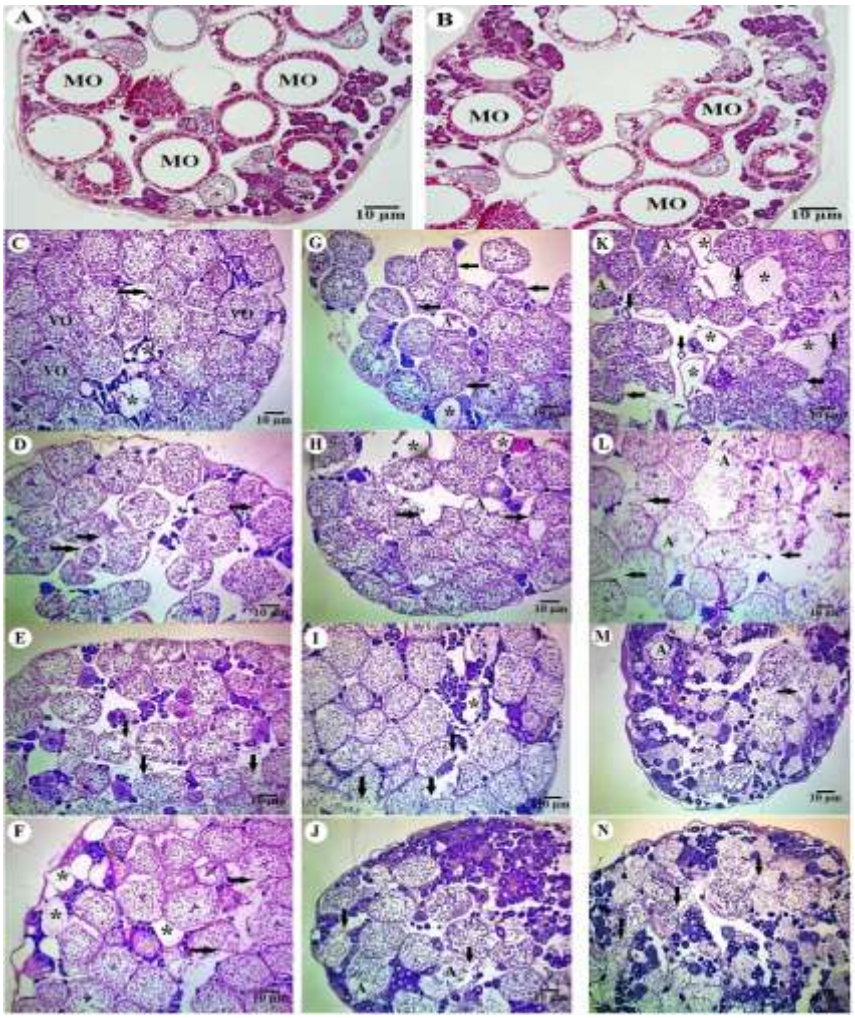


Fig. 24 Photomicrographs showing spawning ovary of *Anabas testudineus* exposed to triclosan. A-Control, MO-Mature oocyte; B-Vehicle, MO-Mature oocyte; C-F: Triclosan-exposed (0.009 µg L^{-1} for 4, 7, 30 and 60 d, respectively); G-J: Triclosan-exposed (9 µg L^{-1} for 4, 7, 30 and 60 d, respectively); K-N: Triclosan-exposed (176.7 µg L^{-1} for 4, 7, 30 and 60 d, respectively); VO-Vitellogenic oocyte, Asterisks (*)-Empty follicle (C, F, G, H, I, K), Arrow-Oocyte membrane damage (C, D, E, F, G, H, I, J, K, L, M, N), A-Follicular atresia (G, H, J, K, L, M), Arrow (↓)-Aneucleated early stage oocytes (K)

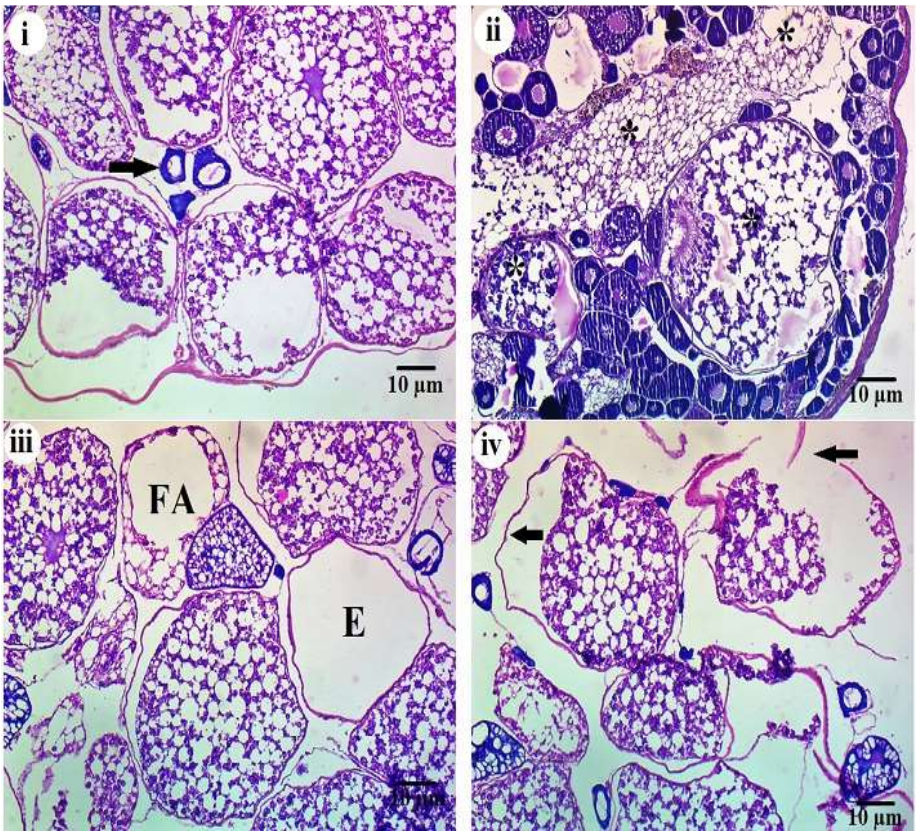


Fig. 24a **Photomicrographs showing representative pathologies of spawning ovary in *Anabas testudineus* exposed to triclosan**. i - Anucleated perinucleolus oocyte (→); ii - Degenerated oocytes (*); iii - Follicular atresia (FA), Empty follicle (E); iv - Oocyte membrane damage (←)

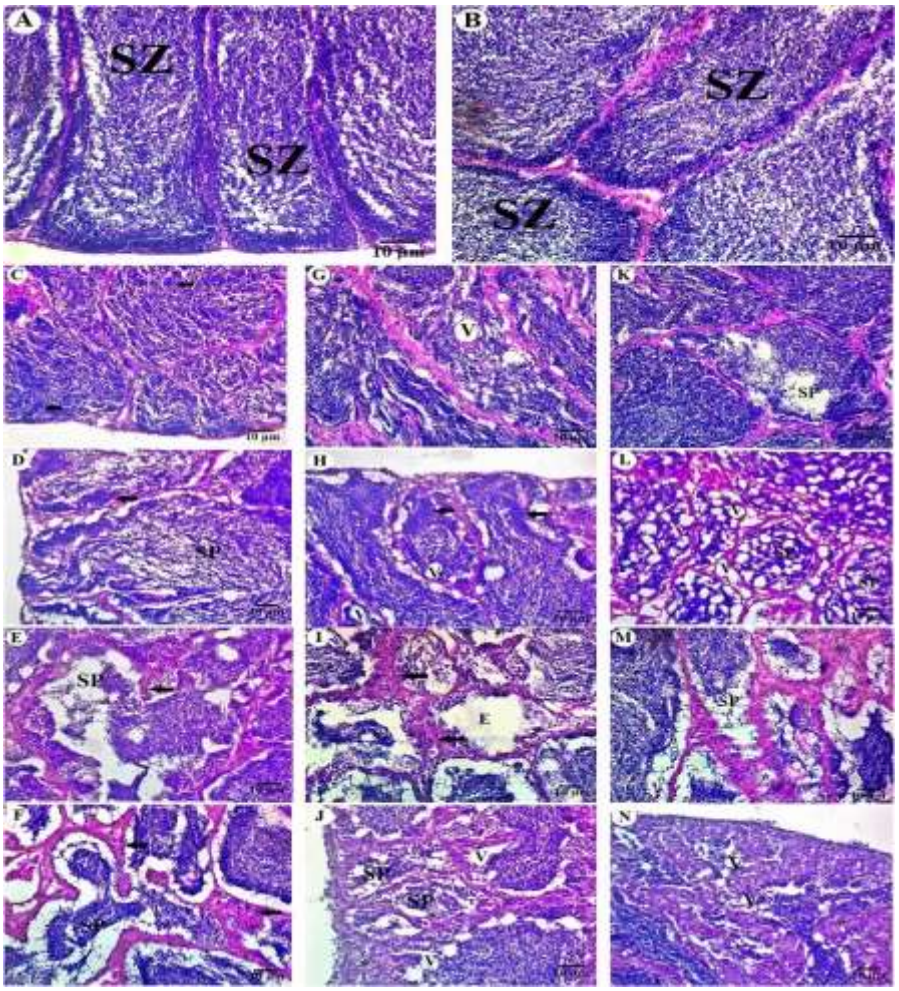


Fig. 25 **Photomicrographs showing testis of *Anabas testudineus* exposed to triclosan**. A-Control, SZ-Spermatozoa; B-Vehicle, SZ-Spermatozoa; C-F: Triclosan-exposed (0.009 µg L^{-1} for 4, 7, 30 and 60 d, respectively); G-J: Triclosan-exposed (9 µg L^{-1} for 4, 7, 30 and 60 d, respectively); Triclosan-exposed (176.7 µg L^{-1} for 4, 7, 30 and 60 d, respectively); SP-Loss of spermatozoa (D, E, F, J, K, L, M), Arrow (←) – Spermatocyst (C, D, H), Arrow (←) – Disorganised and thickened seminiferous tubule (E, F, I), V-Vacuolization (G, H, J, L, N), E-Empty seminiferous tubule (I), Asterisks (*)-Disorganized seminiferous tubule (M, N)

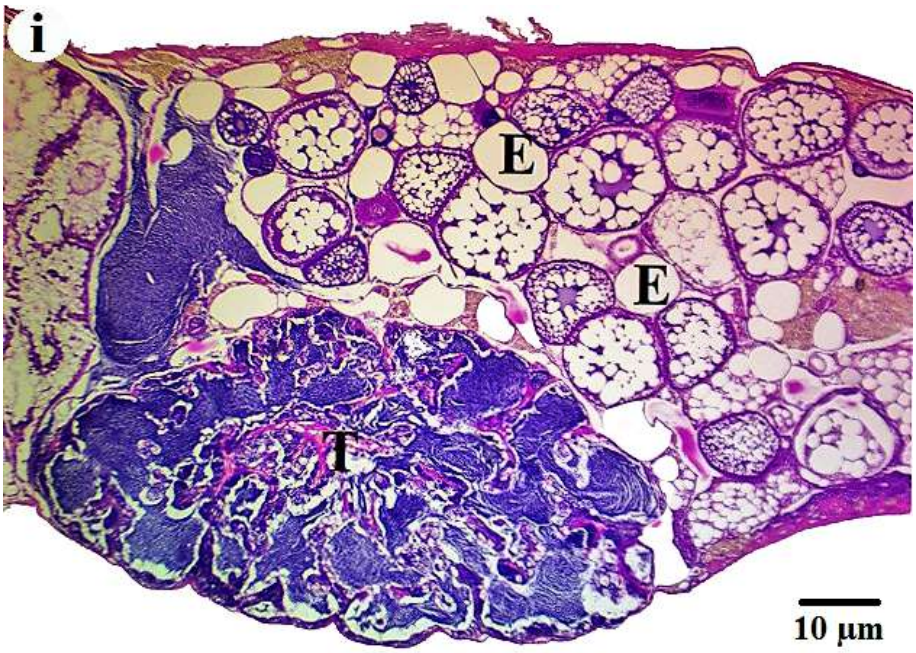


Fig. 25a **Photomicrographs showing development of testis-ova in *Anabas testudineus* exposed to triclosan** (176.7 µg L^{-1} for 60 d), E-Empty follicle, T-Testis

6. DISCUSSION

6.1 Effect of triclosan on the body weight, mucus deposition, and organ weights of the fish in the spawning phase of reproduction

The body weight of the fish, *Anabas testudineus* measured after triclosan exposure at 0.009, 9, and 176.7 µg L^{-1} concentrations for 30 and 60 d during the spawning phase showed a significant reduction in both sex indicating toxicant-related stress. Similar to the pre-spawning period, sublethal triclosan exposure groups caused a remarkable reduction in the body weights, and this could be due to a reduction in food consumption or anorexia in fish. Estrogenic compounds are usually anorexigenic, which inhibit the daily food intake thereby reduces the body weight of animals (Brown and Clegg, 2010). A similar reduction in body weight has been observed after exposure to ethinylestradiol at environmentally relevant concentrations in male and female *Oreochromis niloticus* (Shved *et al*., 2008). Exposure of phthalate esters to zebrafish and *Pseudetroplus maculatus* also caused a similar reduction in the body weight associated with a decrease in daily food intake (Kim *et al*., 2015; Sajla *et al*., 2019).

The present study observed that triclosan exposure increased the mucous deposition in the fish as the defensive mechanism against the toxicant. Some of the chemical stressors including phthalate plasticizers and nanomaterials have been shown to increase the mucus deposition in the fish *Pseudetroplus maculatus* and *Anabas testudineus*, respectively (Sruthi *et al*., 2020; Sumi and Chitra, 2020).

The absolute and relative weights of liver and gonadal tissues showed a remarkable reduction after 30 and 60 d of triclosan exposure, which indicated the failure of metabolic activity and gonadal development in the fish. The results were in agreement with other findings that on exposure to some xenoestrogens such as 17α-ethinylestradiol to the adult fathead minnow, *Pimephales promelas* (Filby *et al*., 2007), dibutyl phthalate to *Pseudetroplus maculatus* (Sruthi *et al*., 2020), sodium benzoate to *Anabas testudineus* (Vijayakumar *et al*., 2020), and malathion to *Channa punctatus* (Bharti and Rasool, 2021) have been shown to alter the absolute and relative weights of liver and gonadal tissues.

6.2 Effect of triclosan on the level of total protein in the plasma, gonads, and liver tissues

Plasma protein consisting of albumin, globulin, and fibrinogen are synthesized majorly in the liver and a small percentage by lymphocytes and plasma cells. Plasma protein has been known to involve in the control of several physiological activities, and therefore, used as a diagnostic tool to detect diseases, or the overall health status of the animal (Cnaani *et al*., 2004). The present study observed that triclosan exposure elevated the level of total protein in plasma to meet the energy demand. However, the level of total protein in the gonads and liver tissues declined thereby indicated the suppression of several biological processes. The results suggested that triclosan exposure caused degradation and release of tissue protein into the circulation as an alternate source of energy to overcome the stress condition, and also

as an attempt to detoxify the toxicant. Similar observations have been reported after exposure of the estrogenic environmental contaminants namely phorate and chlorpyrifos to *Channa punctatus* and *Pseudetroplus maculatus*, respectively (Singh *et al*., 2010; Raibeemol and Chitra, 2018).

6.3 Effect of triclosan on the level of vitellogenin in blood plasma, gonads, and liver tissues

Vitellogenin is a phospholipoglycoprotein synthesized and secreted by the liver of female oviparous fish in response to estrogen stimulus on binding to the nuclear estrogen receptors (Nagler *et al.*, 2010). Vitellogenin is transported through the bloodstream, and incorporated into the growing oocytes, where it is processed and stored as yolk proteins in the ooplasm (Arukwe and Goksoyr, 2003; Hennies *et al*., 2003). The concentration of the protein is high in the mature female fish since it acts as a precursor of egg-yolk protein in the developing oocytes. However, in the male liver, the gene for vitellogenin protein remain unexpressed until it gets activated by the estrogen mimics (Denslow *et al*., 1999), which indicate the gender-specific production of vitellogenin, and difference in the sensitivity and expression of estrogen receptors (Navas and Segner, 2006). Thus the elevated level of vitellogenin is considered as the biomarker for the estrogenic effects of xenobiotics in aquatic organisms (Nagler *et al*., 2010).

The present study proved the estrogenic effects of triclosan in female fish by the induction of vitellogenin levels in blood plasma and

liver while ovarian tissue showed a decline in the level of vitellogenin, which suggested the failure in the deposition of egg yolk protein possibly due to the low level of FSH (Tyler *et al*., 1991). Similarly, in another study triclosan has been shown to induce the level of liver vitellogenin in female carp, *Cyprinus carpio* by binding to the estrogen receptor in liver tissues thereby proved the estrogenicity of the compound (Wang *et al*., 2017).

The present study observed an elevated level of vitellogenin in the plasma, testis, and liver tissues of male fish, which further confirmed that an increase in the level of serum estradiol positively regulated the synthesis of vitellogenin from the liver tissues. Another study supported the present findings that the exposure of triclosan at 20 and 100 mg L^{-1} concentrations for 21 d had increased the production of vitellogenin in liver tissues of male medaka (Ishibashi *et al*., 2004). Similarly, exposure of triclosan at 0.08 and 016 mg L^{-1} concentrations to juvenile male Yellow River carp for 42 d increased vitellogenin levels in the hepatopancreas, which has been mediated through non-estrogen receptor signaling pathway like up-regulation of aromatase expression and elevated levels of endogenous estradiol thereby proved anti-androgenic effects of the compound (Wang *et al*., 2018). Several studies have documented that the effects of estrogenic compounds such as atrazine, chlordecone, 17β-estradiol, ethinylestradiol, and dibutyl phthalate on the induction of vitellogenin level in male fishes like *Poecilia sphenops, Oreochromis niloticus, Geophagus brasiliensis,* and *Pseudetroplus maculatus*, respectively (Vasanth *et al*., 2015; Chen

et al., 2019; Yamamoto *et al*., 2017; Asifa and Chitra, 2019; Sruthi *et al*., 2020).

6.4 Effect of triclosan on the levels of serum hormones

Estradiol and testosterone are the potent female and male sex hormones involved in the reproduction of fish. The secretion of sex hormones is influenced by the gonadotropins namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the anterior pituitary, which is produced by the gonadotropin-releasing hormone of the hypothalamus (Munakata and Kobayashi, 2010). Besides, thyroid-stimulating hormone (TSH) is also involved in the maintenance of normal reproduction and other metabolic activities in fish (Raine, 2011). The study evaluated the effect of triclosan on sex hormones, gonadotropins, and TSH in both female and male fish to assess the endocrine-disrupting effects of the compound.

Female fish exposed to triclosan showed a reduction in the level of estradiol and testosterone along with a decline in the levels of FSH and LH stating the disruption at the hypothalamic-pituitary-gonadal (HPG) axis. The results were in agreement with another study that on triclosan exposure to the female yellow river carp, *Cyprinus carpio* resulted in the decline of sex steroid hormones and gonadotropins thus proved estrogenicity of the toxicant (Wang *et al*., 2017). In another study, a similar reduction in the level of sex steroids and gonadotropins in serum of the fish *Oreochromis mossambicus* has been observed after exposure to phthalate plasticizers (Revathy and Chitra, 2019).

Male fish exposed to triclosan showed an elevated level of estradiol after 60 d of sublethal treatment while the level of serum testosterone along with FSH and LH decreased in all exposure groups, which demonstrated anti-androgenic effects of the compound. The results clearly illustrated that the mechanism of endocrine disruption was mediated through the non-estrogen receptor-signaling pathway, i.e., by aromatization that converts testosterone to estradiol. The inhibition in the activities of testicular steroidogenic enzymes lowered the level of testosterone, which in turn could have negatively regulated the secretion of gonadotropins in the male fish. The findings were in concordance with another study, in which di-(2-ethylhexyl)-phthalate (DEHP) exposed to adult male zebrafish increased estradiol but declined testosterone level associated with the up-regulation of aromatase gene expression and inhibition of spermatogenesis (Ma *et al*., 2018).

Triclosan exposure caused a remarkable rise in the level of TSH in blood serum of both female and male fish suggesting hyperplasia of thyroid glands, which could have stimulated the release of hormone from the anterior pituitary thereby affects the thyroid functions and reproductive potential of the fish. Some studies have documented that exposure of endocrine disruptors such as ammonium perchlorate and triclosan to fathead minnows, *Pimephales promelas,* and zebrafish, respectively, caused thyroid hyperplasia with an increased follicular epithelial cell height and reduced colloid thereby resulted in elevated TSH level (Crane *et al*., 2005; Pinto *et al*., 2013). Altogether, the present findings illustrated that interference of triclosan

with the HPG axis and thyroid axis could have resulted in the reduction of overall reproductive functions in the fish.

6.5 Effect of triclosan on the activities of gonadal steroidogenic enzymes

The gonadal steroidogenic enzymes including 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) are primarily involved in the synthesis of sex steroid hormones under the influence of pituitary gonadotropins (Villeneuve *et al*., 2007; Rasmussen *et al*., 2013). In the present study, triclosan exposure caused a reduction in the activities of 3β-HSD and 17β-HSD enzymes in gonads of both female and male fish indicating dysregulation of gonadal steroidogenesis, which ultimately lead to altered biosynthesis of sex steroid hormones as explained in the previous section. The inhibition in the activities of the key steroidogenic enzymes in gonads represented endocrine-disrupting effects of the toxicant, which could induce reproductive dysfunction in fish (Villeneuve *et al*., 2008).

The findings coincided with a previous study which demonstrated that exposure to phthalate plasticizers such as di-isononyl-phthalate (DINP) and di-(2-ethylhexyl)-phthalate (DEHP) has been shown to reduce the activities of 3β-HSD and 17β-HSD enzymes in the gonads of the freshwater fish, *Oreochromis mossambicus* (Revathy and Chitra, 2019). Another study on the exposure of bisphenol A to rare minnow *Gobiocypris rarus* has been shown to decline the activities of endogenous key steroidogenic enzymes

mediated through the abnormal DNA and histone methylation (Liu *et al.*, 2020). In a recent study, Indian catfish *Heteropneustes fossilis* and Indian major carp *Cirrhinus cirrhosus* treated with sodium dodecyl sulfate for 30 d decreased the activities of 3β-HSD and 17β-HSD enzymes irrespective of sexes in the spawning phase thereby proved interruption in the normal steroidogenesis followed by impaired sex hormone production (Moniruzzaman and Saha, 2021).

6.6 Effect of triclosan on fecundity and sperm parameters

The reproductive performance of *A. testudineus* during the spawning period was analyzed using some reproductive indices such as rate of fecundity in females, and sperm parameters like viability, motility, and sperm count in males. The reduction in sperm indices after triclosan exposure suggested the failure of reproductive performance in male fish. An investigation conducted in the Lake Mead National recreational area has reported that triclosan along with other environmental contaminants altered sperm quality parameters such as motility, viability, mitochondrial membrane potential, sperm count, sperm morphology, and DNA fragmentation in male common carp, *Cyprinus carpio* (Jenkins *et al*., 2018).

The present study observed that triclosan exposure resulted in a remarkable decline in the fish fecundity at all concentrations after 30 and 60 d. Another study has reported that the larval exposure to environmentally relevant concentrations of triclosan impaired fecundity and fertility in adult zebrafish (Stenzel *et al*., 2019). The present findings were in agreement with a recent study that Japanese

medaka on exposure to DEHP for 21 d during early developmental stage has been shown to reduce egg production in adult females, and also caused a significant decline in the mature sperm count in adult male fish thereby resulted in an irreversible reproductive impairment (Yuen *et al*., 2020).

6.7 Effect of triclosan on the histology of gonads

Histology is the powerful tool routinely used in toxicology to study the reproductive health of fishes associated with the effects of endocrine-disrupting chemicals. Histology of the ovary performed during the spawning phase of the fish showed remarkable lesions in all triclosan exposure groups, where the severity of damage increased with duration and concentration. The most prominent histological alterations observed in the ovary include degenerated oocytes with membrane damage, empty and atretic ovarian follicles, formation of anucleated perinucleolus oocyte, and decline in the number of vitellogenic oocytes.

Similar observations have been reported in the fathead minnow exposed to fungicides thereby documented the reproductive abnormalities (Ankley *et al*., 2005). The degenerated oocytes and atretic follicles observed in the present study were found correlated with the reduction in the levels of gonadotropins that are essential for oocyte maturation during the spawning phase of fish. The decline in the expression of gonadotropin genes associated with suppression of oocyte growth has been documented after bisphenol A and estradiol exposure in zebrafish (Chen *et al.*, 2019). *Pseudetroplus maculatus*

exposed to dibutyl phthalate has been shown to cause severe morphological ovarian damages such as empty follicles, atretic oocytes, membrane blebbing, nuclear condensation, vacuolization, and broken theca granulosa membrane, which are associated with apoptosis in oocytes of the fish (Sajla *et al*., 2019). The overall alterations in the histoarchitecture of ovarian tissue after triclosan exposure indicated synergistic estrogenic effects of the toxicant in *A. testudineus*.

Triclosan also caused remarkable testicular damages as indicated by severe pathologies like loss of spermatozoa, formation of spermatocyst, disorganized, thickened, and empty seminiferous tubules, and vacuolization that accounts for the reduction in sperm motility, viability, and sperm count in the exposed fish. The seminiferous tubules containing early spermatogenic cells and interstitial thickening along with a reduced number of spermatozoa in the spawning stage of testis revealed triclosan exposure altered spermatogenesis. The results coincided with another study that on exposure of goldfish to bisphenol A caused irreversible changes in spermatogenesis (Wang *et al*., 2019). The mature male Japanese medaka exposed to DEHP during the early development stage had observed an increase in interstitial space followed by a decline in sperm count (Yuen *et al*., 2020). Similar observations have been reported in the fish *Pseudetroplus maculatus* exposed to dibutyl phthalate suggesting altered reproductive performance that ultimately lead to infertility in male fish (Sruthi *et al*., 2020).

The present study observed the development of testis-ova in the fish after exposure of triclosan at 176.7 µg L^{-1} concentration for

60 d, which represents the formation of intersex. Likewise, male Japanese medaka exposed to triclosan and anti-androgenic *p,p'*-DDE has been shown to induce the formation of gonadal intersex (Sun *et al*., 2016; Wang *et al*., 2018). The present findings suggest that triclosan exposed during the spawning phase influenced the reproductive physiology of *A. testudineus*, where the concentration of the toxicant and duration of exposure plays a significant role.

7. CONCLUSIONS

1. Triclosan exposure at environmentally relevant and sublethal concentrations during the spawning period of *Anabas testudineus* increased the level of total plasma protein and declined in gonad and liver tissues, which indicated the degradation and release of tissue protein into the circulation as an alternate source of energy to overcome the stress condition.
2. The estrogenicity of triclosan was proved by the induction of vitellogenin in blood plasma, liver, and testis, while low levels of vitellogenin in the ovarian tissue denoted the failure of egg yolk protein deposition into the oocytes.
3. Triclosan down-regulated the secretion of sex steroid hormones and gonadotropins in female fish by acting on the hypothalamic-pituitary-gonadal axis.
4. Male fish exposed to triclosan showed elevated estradiol and vitellogenin levels with a decreased serum testosterone and gonadotropins, representing anti-androgenic effects of the compound mediated through the non-estrogen receptor-signaling pathway, i.e., aromatization of testosterone into estradiol.
5. The rise in the level of TSH in the blood serum of both female and male fish suggested hyperplasia of thyroid glands, which stimulated the anterior pituitary to release the hormone, and disrupted normal thyroid functions and reproductive potential in the fish.

6. Triclosan altered gonadal steroidogenesis as evident by the reduction in the activities of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase enzymes in gonads, which ultimately lead to the altered biosynthesis of sex steroid hormones.
7. Decline in the fecundity of female fish and the reduction in sperm motility, viability, and count of male fish suggested the failure of reproductive performance.
8. Triclosan caused remarkable lesions in the ovary, which included degenerated oocytes with membrane damage, empty and atretic ovarian follicles, formation of anucleated perinucleolus oocyte, and decline in the number of vitellogenic oocytes.
9. Histology of testis showed severe pathologies such as loss of spermatozoa, formation of spermatocyst, disorganized, thickened and empty seminiferous tubules and vacuolization in the spawning phase, which possibly lead to male infertility.
10. The development of testis-ova in triclosan-exposed fish represents the formation of intersex.
11. Overall, the study concluded that exposure to triclosan during the spawning period affected the normal physiology of reproduction in the fish, *A. testudineus*.

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CHAPTER 6

Assessment of triclosan-induced oxidative stress and genotoxicity as biomarkers in the fish, *Anabas testudineus*

Chapter 6 Assessment of triclosan-induced oxidative stress and genotoxicity as biomarkers in the fish *Anabas testudineus*

CONTENTS

LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µL	Microlitre
µg L^{-1}	Microgram per litre
•OH	Hydroxyl radical
8-OHdG	8-hydroxy-2'-deoxyguanosine
ANOVA	Analysis of Variance
APHA	The American Public Health Association
ATP	Adenosine triphosphate
CAS No.	Chemical Abstracts Service Number
Cu-Zn SOD	Copper-Zinc-dependent Superoxide dismutase
CYP1A	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP3A	Cytochrome P450, family 1, subfamily 3A, polypeptide 1
d	Days
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DTNB	Di-thionitro-benzoic acid
EC-SOD	Extracellular Superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
Fe^{2+}	Ferrous ion
g	Centrifugal force
GR	Glutathione reductase
GSH	Reduced glutathione
GSSH	Oxidized glutathione
GST	Glutathione S-transferase
H_2O	Water
H_2O_2	Hydrogen peroxide
HPG	Hypothalamic-pituitary-gonadal axis
hsc70a	Heat shock proteins 70a gene
hsp90ba	Heat shock proteins 90 ba gene
hsp90bb	Heat shock proteins 90 bb gene

K^+	Potassium ion
L	Litre
$L^{\bullet}$	Lipid radical
LC_{50}	Median lethal concentration
$LOO^{\bullet}$	Lipid peroxy radical
LOOH	lipid hydroperoxide
M	Molarity
MDA	Malondialdehyde
mg L^{-1}	Milligram per litre
min	Minutes
miR-200b-5p	MicroRNA 200b-5p gene
miR-21	MicroRNA 21 gene
miR-34b	MicroRNA 34b gene
mL	Millilitre
mM	Millimolar
Mn-SOD	Manganese-dependent Superoxide dismutase
N	Normality
Na^+	Sodium ion
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
nm	Nanometer
nmol/ nM	Nanomol
Nrf2/ARE	Nuclear factor erythroid 2-related factor 2/ Antioxidant response element
$O_2^{\bullet-}$	Superoxide radical
pH	Concentration of hydrogen ions
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SD	Standard Deviation
Sirt1	Silent information regulator-1 or Sirtuin 1 gene
Sirt2	Silent information regulator-2 or Sirtuin 2 gene
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
UV	Ultraviolet
w/v	Weight by volume

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1. INTRODUCTION

1.1 Reactive oxygen species in fish reproduction

The availability of oxygen in the aquatic environment is 33-fold less than in the terrestrial environment, which is influenced by several factors including temperature, salinity, pH, presence of pollutants, etc. In aquatic organisms, about 80-95% of the oxygen consumed is later reduced to water by the mitochondrial electron transport system. Among the consumed oxygen, 3-5% is leaked by a reduction of an electron from the ground state of an oxygen molecule to form a superoxide ($O_2^{\bullet-}$) radical (Turrens *et al*., 1985). According to 'the superoxide theory of oxygen toxicity', the formation of superoxide is considered as one of the major factors in oxygen toxicity. The sequential addition of electrons to superoxide forms hydrogen peroxide, which is the most reactive hydroxyl radical that cause damage to the cell even at relatively low concentration (Storz and Imlay, 1999).

The hydroxyl radical is generated by Haber-Weiss reaction and Fenton reaction where it results in the partial reduction of oxygen to form reactive chemical intermediates known as free radicals or reactive oxygen species (ROS). Free radicals are the chemical species like atoms, molecules, or ions with one or more unpaired electrons in their valence shell (Halliwell and Gutteridge, 1999; Phaniendra *et al*., 2015). The odd numbers of electrons in the free radicals are unstable, short-lived, and highly reactive so that they combine with other compounds to capture electrons for attaining stability, which is referred to as 'stealing of electron' (Kelly, 2003). The attacked molecule becomes a

free radical by losing its electron where a chain reaction cascade leads to the disruption of a living cell (Mukherji and Singh, 1986).

ROS are free radicals consisting of singlet oxygen, superoxide anion, hydroxyl, peroxyl, hydroperoxyl, lipid peroxyl, alkoxyl radicals, nitric oxide, nitrogen dioxide, and their derivatives, and also includes non-radicals such as hydrogen peroxide, hypochlorous acid, and ozone, which are relatively more reactive than molecular oxygen. The excess of ROS that is continuously generated in the biological systems disrupts the redox status thereby leading to oxidative or reductive stress (Lushchak, 2011). ROS also act as second messengers in physiological cell signaling and therefore have an important role in developmental and reproductive processes such as embryonic development, cell proliferation, and differentiation (Agarwal *et al*., 2012).

During oocyte maturation, hydrogen peroxide generation is regulated by the inactive state of mitochondria and low oxygen metabolism thereby results in a low risk of DNA mutation in the developing oocytes. However, excess ROS levels contribute to spindle instability, chromosomal abnormalities, and oocytes aging (Sasaki *et al*., 2019). ROS generation also plays a key role in altering the male reproductive functions such as sperm fertilizing ability, acrosome reaction, hyperactivation, and sperm-oocyte fusion by acting on the hypothalamic-pituitary-gonadal (HPG) axis (Darbandi *et al*., 2018). High levels of ROS induce oxidative stress and impair testicular functions namely spermatogenesis, steroidogenesis, epididymal sperm maturation, alteration in sperm parameters like sperm viability,

motility, and sperm counts thereby lead to infertility (Aitken, 2017). Therefore, the surplus of ROS generated by exogenous or endogenous factors is maintained by the normal functioning of the antioxidant defense system, which is crucial for normal reproductive functions (Aitken, 2019).

1.2 Antioxidant defense system

All aerobic organisms, including fish, have integrated enzymatic and non-enzymatic antioxidant defense systems (Livingstone, 2001). The low molecular mass non-enzymatic antioxidants include water-soluble compounds like reduced glutathione, ascorbic acid, and lipid-soluble compounds such as carotenoids, retinol, α-tocopherol, urate, and retinyl esters (Das *et al*., 2018). The high molecular mass antioxidants are generally enzymatic, which includes antioxidant enzymes such as superoxide dismutase, catalase, selenium-dependent glutathione peroxidase, DT-diaphorase, glutathione reductase, glucose-6-phosphate dehydrogenase, etc. Besides, some non-specific high molecular mass antioxidants like metallothioneins and ferritin are known to bind with iron and copper metals to prevent ROS-induced damage (Valavanidis *et al*., 2006).

Ascorbic acid or vitamin C preferentially reacts with radicals directly in the aqueous phase of the cells to protect the cellular organelles from free radical damage (Beyer, 1994; Hossain *et al*., 2018). Vitamin E or α-tocopherol is a lipid-soluble antioxidant that scavenges the free radical against lipid peroxidation thereby protects cell membranes from oxidative stress (Yamauchi, 1997). Retinoids, the

animal-based and carotenoids, the plant-based vitamin A derivatives are the potent lipid-soluble antioxidant capable of quenching singlet oxygen (Dao *et al*., 2017).

Superoxide dismutase (SOD) exists in different isoforms such as copper-zinc-dependent SOD (Cu-Zn-SOD), manganese-dependent SOD (Mn-SOD), and extracellular SOD (EC-SOD). SOD is a primary metalloenzyme involved in the antioxidant defense system that converts superoxide radical ($O_2^{\bullet -}$) into molecular oxygen and hydrogen peroxide.

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Catalase is the tetrameric heme-containing enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2) and protects the cellular organelles from peroxide damage.

$$2H_2O_2 \rightarrow H_2O + O_2$$

The most reactive radical formed from superoxide and hydrogen peroxide is hydroxyl radicals (HO^-), which is a harmful ROS but has a very short half-life (Schieber and Chande, 2014). The hydroxyl radical is also formed from hydrogen peroxide through Haber-Weiss and Fenton reactions (Dong *et al*., 2017). The decomposition of hydrogen peroxide occurs in a chain of reaction by initiation, propagation, and termination, where the chain is initiated by Fenton reaction as follows:

$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$ ……Initiation reaction (Step 1)

The propagation of reaction occurs in two steps as follows:

$H_2O_2 + {}^{\bullet}HO \rightarrow O^{2\bullet -} + H^+ + H_2O$ ……Propagation reaction (Step 2a)

$H_2O_2 + O^{2\bullet -} + H^+ \rightarrow {}^{\bullet}HO + O_2 + H_2O$….Propagation reaction (Step 2b)

The hydroxyl radical formed is scavenged by ferrous ions in the termination reaction as follows:

$Fe^{2+} + {}^{\bullet}HO + H^{+} \rightarrow Fe^{3+} + H_2O$ ……..Termination reaction (Step 3)

The glutathione reductase/peroxidase system catalyzes the reduction of H_2O_2 into water molecules by the conversion of reduced glutathione into oxidized form (Hoseinifar *et al*., 2020). The glutathione system constitutes the non-enzymatic second-line antioxidant defense system, which includes antioxidant molecules such as reduced glutathione (GSH), oxidized glutathione (GSSH), and glutathione-related enzymes like glutathione reductase, glutathione peroxidase, and glutathione S-transferase. The redox reactions are maintained in the cell through the glutathione system in the presence of selenium where the reduced form is converted into the oxidized form of glutathione in the presence of glutathione peroxidase enzyme, which protects the cells from membrane damages (Monteiro *et al*., 2006). The glutathione reductase enzyme in the presence of riboflavin revitalizes glutathione, which is previously oxidized by the action of the glutathione peroxidase enzyme (Parolini *et al*., 2019).

The highly reactive free radicals formed attacks proteins, lipids, and DNA thereby imbalance the redox status of the cell leading to oxidative stress (van der Oost *et al*., 2003). The oxidative degradation of lipids in the cell membrane resulting in cell damage is referred to as lipid peroxidation (Ayala *et al*., 2014). The carbonylation of protein includes oxidative modifications of protein resulting in the formation of carbonyl groups namely aldehyde or ketone groups, which is widely used as an indicator of oxidative damage (Weng *et al*., 2017). The

excess of free radicals also contributes to the induction of oxidative DNA damage in the ovarian epithelium and sperm membrane that plays a key role in the pathogenesis of both female and male reproduction in fish (Agarwal *et al*., 2012; Aitken *et al*., 2014).

1.3 Biomarkers of oxidative stress

Biomarkers are any characteristics measured as an indicator of normal biological processes in response to a toxicant or any chemical exposure and serve as an early warning signal to diagnose the health status of an organism (Califf, 2018). Most of the components of the antioxidant system and oxidative byproducts are recommended as biomarkers of oxidative stress in fish. It includes the activities of antioxidant enzymes (Doyotte, 1997; Pandey *et al*., 2003), non-enzymatic antioxidants (Parvez *et al*., 2006), generation of hydrogen peroxide (Tkachenko *et al*., 2013), lipid peroxidation (Yoshida *et al*., 2013), and protein oxidation (Parvez and Raisuddin, 2005). The free radicals formed as a result of ROS production also lead to DNA damage that is usually detected using micronucleus formation and comet assay (Beedanagari *et al*., 2014; Hussain *et al*., 2018). The disturbances in cellular oxidative homeostasis eventually damage DNA (Lushchak, 2016). Hence oxidative stress and genotoxicity are together evaluated as valuable biomarkers to monitor water quality in freshwater ecosystems.

1.3.1 Lipid peroxidation - an oxidative degradation of lipids

Lipid peroxidation is a chain of reaction in which the oxidants such as non-radical or free radical species attack the membrane phospholipids, especially polyunsaturated fatty acids (PUFAs) resulting in the formation of alkanes, aldehydes, carboxylic acids, ketones, and polymerization products thereby lead to oxidative damage (Yin *et al*., 2011). A set of arachidonic acid oxidation products called isoprostanes is used as the valid biomarker of lipid peroxidation. The oxidation of unsaturated fatty acids also yields short-lived lipid hydroperoxides, which then react with metals and produce highly reactive epoxides and aldehyde. Malondialdehyde (MDA) is one of the important aldehyde products of lipid peroxidation, which is mutagenic and carcinogenic in both terrestrial and aquatic organisms (Pourahmad *et al*., 2016).

Lipid peroxidation occurs in three main steps as initiation, propagation, and termination thereby resulting in the formation of non-radical products.

In the initiation step, a carbon-centered lipid radical ($L^{\bullet}$) is formed from a hydroxyl radical.

$LH + R^{\bullet}$ or $HO^{\bullet} \rightarrow L^{\bullet} + RH$ or HOH ……Initiation reaction (Step 1)

In the propagation phase, the lipid radical ($L^{\bullet}$) rapidly reacts with oxygen to form a lipid peroxy radical ($LOO^{\bullet}$).

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ ……..……Propagation reaction (Step 2)

In the termination reaction, lipid peroxy radical abstracts hydrogen from another lipid molecule forming new lipid radical ($L^{\bullet}$) and lipid hydroperoxide (LOOH), a non-radical product.

$LH + LOO^{\bullet} \rightarrow L^{\bullet} + LOOH$ ……Termination reaction (Step 3)

The non-radical lipid hydroperoxide (LOOH) are easily converted into highly reactive species such as lipid alkoxyl radicals, aldehydes such as malondialdehyde, alkanes, lipid epoxides, and alcohols. Most of the degradation products are mutagens forming DNA adducts and induce altered gene expression. The rate of lipid peroxidation or oxidation of polyunsaturated fatty acids in the cells is measured using several endpoints such as malondialdehyde, thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides, hexanal, propanal, and 4-hydroxynonenal (Livingstone, 2001; Ayala *et al*., 2014).

1.3.2 Protein carbonylation - an oxidative damage of proteins

Carbonylation is an irreversible, non-enzymatic modification of proteins resulting in unfolding or alteration of protein structure. The interaction of proteins with free radicals induces certain post-translational modifications including alteration of amino acid side chains, ROS-mediated peptide cleavage, reactions of peptides with lipid and carbohydrate oxidation products, and production of protein carbonyl derivatives. Protein carbonylation is widely used as a biomarker for protein oxidation induced by ROS in biological systems (Hematyar *et al*., 2019). Protein carbonylation is also stimulated by lipid peroxidation end products such as malondialdehyde and unsaturated aldehydes, which cause covalent, irreversible modification of the side chains of cysteine, histidine, and lysine residues of proteins,

resulting in the formation of carbonyl derivatives and finally protein degradation (Davies and Dean, 1997).

ROS generated during oxidative stress damages the peptide backbone, resulting in the generation of protein carbonyls, where the alpha-carbon in the peptide chain loses hydrogen to form protein radicals. The two protein radicals that lie nearby undergo cross-links to form peroxide intermediates, leading to rearrangement, and subsequent cleavage of the peptide bond forms carbonyl-containing peptides (Davies *et al*., 1995). Aromatic amino acids are highly susceptible to oxidation thus producing phenoxyl radicals, which are irreversible reductants (Hawkins and Davies, 2001). However, aliphatic amino acids that lack sulphur are oxidized by hydrogen abstraction at the α-carbon generating a carbon-centered radical (Stadtman, 1993).

Proteins are highly susceptible to the free radical attack in cells by oxidative modifications (Du and Gebicki, 2004). The formation of protein carbonyls increases susceptibility to proteolysis by degradation of specific proteases, and fragmentation of the peptide chain (Dalle-Donne *et al*., 2003). High levels of protein carbonyl contents are found associated with several human pathologies including Alzheimer's disease, rheumatoid arthritis, diabetes, sepsis, chronic renal failure, and respiratory distress syndrome (Fedorova *et al*., 2014).

1.3.3 Oxidative DNA damage

The excessive production of ROS potentially causes DNA damage in both mitochondria and nucleus by the formation of single- or double-stranded DNA breaks, base modification, sugar damage, and

DNA-protein cross-links (Jena, 2012). The hydroxyl radicals formed through the Fenton reaction are mainly involved in DNA damage, where it reacts with all components of the DNA molecule such as the pyrimidine and purine bases, and sugar moiety leading to oxidative genetic damage (Dizdaroglu *et al*., 2002). The DNA damages subsequently lead to genotoxicity by the induction of micronuclei formation, chromosomal and nuclear abnormalities, gene mutations, frameshifts, deletions or translocations, chromosomal rearrangements, and alteration of signal transduction (Vieira *et al*., 2018).

Hydroxyl radicals attack pyrimidines at the C5 and C6 site of cytosine and thymine forming C5-OH- and C6-OH-adduct radicals. Oxygen added to C5-OH-adduct radicals produces 5-hydroxy-6-peroxyl radicals that scavenge superoxide by reacting with water forming cytosine and thymine glycols (Pourahmad *et al*., 2016). However, hydroxyl radicals attack purines at C4, C5, and C8 forming respective adduct radicals. Oxido-reduction of C8-OH-adduct radicals produces formamidopyrimidines, 8-hydroxypurines, and 8-oxo-deoxyguanosine, which are used as a potential biomarker of oxidative DNA damage and carcinogenesis (Kelly *et al*., 2008). Hydroxyl radical also reacts with the sugar moiety forming strand breaks. Some altered sugars that remain in the DNA backbone constitute the alkali-labile sites, which are used as biomarkers of hydroxyl-induced DNA-sugar damage products (Hemnani and Parihar, 1998).

1.4 Relevance of the study

The excess of oxygen free radicals generated under exposure to aquatic environmental toxicants may contribute to genetic damage in the aquatic organisms. Hence the role of the antioxidant defense system and its possible mechanisms underlying cellular oxidative damage has become a significant topic of interest in the study of aquatic toxicology. Therefore, the present study was targeted to understand the toxic effects of triclosan, one of the environmental toxicants in the fish, *Anabas testudineus* using oxidative stress and genotoxicity as relevant biomarkers.

2. OBJECTIVE OF THE STUDY

- To assess triclosan-induced oxidative stress and genotoxicity as biomarkers in the fish, *Anabas testudineus*

3. REVIEW OF LITERATURE

Triclosan, the most potent antimicrobial agent has been known to interact with the cell membrane of living organisms and causes damage to the cells through several mechanisms including apoptosis (Wang *et al*., 2020), mitochondrial damage (Raftery *et al*., 2017), immune response (Zhang *et al*., 2019), oxidative stress (Kumar *et al*., 2021), and genetic damage (Hemalatha *et al*., 2019a). The present review emphasis on the toxic effects of triclosan on the biochemical and molecular alterations in antioxidant enzymes and genes involved in the protection against oxidative stress and genetic damages in aquatic organisms, particularly on fish species.

Zebrafish exposed to triclosan during the early life stages as well as in the adult has been found to induce the production of reactive oxygen species along with micronuclei formation (Oliveira *et al*., 2009). Triclosan exposure in the yellow catfish *Pelteobagrus fulvidraco* up-regulated the gene expression of CYP1A, CYP3A, and GST genes with an increase in the activity of catalase enzyme and malondialdehyde level demonstrating the induction of oxidative stress (Ku *et al*., 2014). In a study on the assessment of distribution and bioconcentration of triclosan in fish, *Poecilia vivipara* it has been observed that high accumulation occurred in gonads, followed by liver, gills, brain, and muscle, suggesting that both liver and gonadal tissues as important targets (Escarrone *et al*., 2016). The catfish *Heteropneustes fossilis* exposed to untreated cosmetic effluent

containing triclosan has been shown to increase the activities of antioxidant enzymes like superoxide dismutase and catalase, while the activities of glutathione S-transferase, glutathione peroxidase, and level of reduced glutathione was found to decrease in the liver and gill tissues. Besides, the formation of comet tail in liver and gill cells, and an increased rate of micronuclei in peripheral erythrocytes revealed cyto-genotoxic effects of triclosan in the fish (Banerjee *et al*., 2016).

Exposure of triclosan along with other antimicrobial agents on the fish rainbow trout *Oncorhynchus mykiss* for 40 d has been observed to induce DNA damage in erythrocytes and up-regulated the transcription of *sod, gpx1, gpx2, gsta, hsp90bb, hsp90ba*, and *hsc70a* genes in liver and kidney while down-regulated *cat* genes thereby demonstrated oxidative stress and genotoxicity in the fish (Capkin *et al*., 2017). Sublethal exposure of triclosan to zebrafish at early life stages enhanced glutathione peroxidase activity whereas inhibited the glutathione reductase enzyme, which suggested the *de novo* synthesis of reduced glutathione for the maintenance in the ratio of reduced and oxidized glutathione. The study highlighted that the induction of oxidative stress functions as a major mechanism of neurotoxicity during the developmental period of the fish (Falisse *et al*., 2017).

The elevated levels of cellular oxidative biomarkers such as superoxide dismutase, catalase, glutathione-S-transferase, and Na^+/K^+-ATPase activities in gill and liver tissues of *Pangasianodon hypophthalmus* experimentally infected with dactylogyrid monogenean indicated modulation of innate immune responses and induction of oxidative stress (Kumar *et al*., 2017). Goldfish, *Carassius auratus*

exposed to triclosan for 14 d elevated the levels of malondialdehyde, glutathione, and catalase activity while the reduction in the total antioxidant capacity with subsequent induction of genotoxicity was evident by an increase in the percentage of comet tail moment and occurrence of micronucleus in fish hepatocytes and peripheral blood, respectively (Wang *et al*., 2018). Likewise, in a pH-dependent comparative study on the toxicity of triclosan in five different aquatic organisms, it has been observed that zebrafish was the most sensitive species, while the induction of oxidative stress has occurred in the liver of *C. auratus* under the acidic condition as evident by alteration in the activities of antioxidant enzymes and elevated malondialdehyde level (Li *et al*., 2018).

Topmouth gudgeon *Pseudorasbora parva* exposed to the mixture of fluoxetine and triclosan altered the antioxidant defense system on multiple organs by a prominent increase in the level of lipid peroxidation thereby indicated the induction of oxidative stress (Yan *et al*., 2018). *Pangasianodon hypophthalmus* exposed to triclosan at the sublethal concentration for 45 d has been shown to increase the activities of catalase, glutathione-S-transferase and superoxide dismutase in gill and liver tissues thereby altered enzymatic scavenging properties of enzymes against the free radicals leading to severe physiological alterations (Sahu *et al*., 2018). Exposure of triclosan at environmental concentrations on zebrafish embryos up to 120 hours post-fertilization caused oxidative stress and DNA damage by decreasing the activities of antioxidant enzymes and formation of micronuclei and comet tail (Parenti *et al*., 2019).

Indian major carp *Labeo rohita* exposed to sublethal concentrations of triclosan for 35 d has fluctuated the levels of the enzymatic and non-enzymatic antioxidant index in gill, liver, and kidney tissues along with DNA damage in liver cells using comet assay thereby suggested the induction of oxidative stress and genotoxicity (Hemalatha *et al*., 2019a). Marine flatfish *Solea senegalensis* exposed to triclosan during early development impaired antioxidant system affecting the activity of catalase enzyme at the end of metamorphosis test, however, no oxidative damage on lipids has been detected during the development (Araujo *et al*., 2019).

Pangasianodon hypophthalmus exposed to triclosan at sublethal concentrations for 30 d in varying temperature increased the activities of antioxidant enzymes with a reduction in glutathione-S-transferase enzyme in liver tissues. In addition, severe DNA damage in the nucleus of blood and liver cells, and high micronuclei frequency proved that temperature influence the toxicity of triclosan in the fish (Paul *et al*., 2019). Similarly, in another study, the toxicity of triclosan has been evaluated at different pH such as 6.5, 7.5, and 8.5 on the fish, *P. hypophthalmus,* which showed the induction of oxidative stress at pH 6.5, and has pronounced effects on genetic materials (Paul *et al*., 2020).

Gill and liver tissues of *P. hypophthalmus* exposed to different concentrations of triclosan challenged with a fish pathogenic bacterium *Edwardsiella tarda* has been shown to induce immunosuppression and reduced the survival of fish by the induction of oxidative stress (Bera *et al*., 2020). Sublethal concentrations of triclosan elicited oxidative

stress and DNA damage in the liver and brain tissues of adult zebrafish by decreasing the activities of antioxidant enzymes with a concomitant increase in the level of lipid peroxidation, 8-hydroxy-2'-deoxyguanosine (8-OHdG), and carbonyl protein content (Gyimah *et al*., 2020).

Embryos of silver catfish, *Rhamdia quelen* exposed to environmental concentrations of triclosan, triclocarban, and their binary mixtures for 96 h has altered the activities of antioxidant enzymes where the binary mixtures showed severe toxicity than the single compound (Gomes *et al*., 2021). The up-regulations of *Sirt1*, *Sirt2* genes, and down-regulation of *miR-34b*, *miR-200b-5p*, and *miR-21* genes in the liver tissue has been found to modulate antioxidant system mediated through the Nrf2/ARE signaling pathway by the post-transcriptional regulations in the mosquitofish, *Gambusia affinis* (Bao *et al*., 2021). Zebrafish exposed to sediment spiked with triclosan showed lipid damage and increased superoxide dismutase activity in gill tissue after 2 d, while the increased lipid peroxidation has been observed after 21 d exposure in the liver tissues (Sager *et al*., 2021).

Considering the existing literature, it was found that triclosan induced oxidative stress in various tissues of fish species with relatively less attention on the gonadal tissues. Although the endocrine-disrupting effects of triclosan mediated through the hypothalamic-pituitary-gonadal axis have been well documented, there is paucity in reports describing the mechanism of toxicity. Therefore, in the present study oxidative stress and genotoxicity were evaluated to understand the toxic effects of triclosan in the fish, *Anabas testudineus*.

4. MATERIALS AND METHODS

4.1 Chemicals

Triclosan (CAS No. 3380-34-5; 97% purity), dimethyl sulfoxide (DMSO; 99% purity), thiobarbituric acid, fetal bovine serum, NADPH, ethylenediaminetetraacetic acid (EDTA), di-thio nitro-benzoic acid (DTNB), Giemsa stain, Triton X-100, ethidium bromide, and agarose were obtained from HiMedia Research Laboratories Pvt. Ltd, Mumbai, India. All other chemicals of analytical grade were purchased from the local commercial sources, India.

4.2 Experimental design

Anabas testudineus (8 ± 1 g; 8.5 ± 0.75 cm) of spawning stage randomly selected from the laboratory stock were used.

Groups	**Treatment concentrations n = 10/ group, in replicates**		**Treatment durations**
Control	No toxicant (only tap water)		4, 7, 30, 60 d
Vehicle	Solvent - dimethylsulfoxide (DMSO; 0.001 % v/ v)		4, 7, 30, 60 d
Treatment (Triclosan)	0.009 µg L^{-1}	Environmentally relevant concentration (Ramaswamy *et al*., 2011)	4, 7, 30, 60 d
	9 µg L^{-1}	Environmentally relevant concentration (Nag *et al*., 2018)	4, 7, 30, 60 d
	176.7µg L^{-1}	Sublethal concentration (Priyatha and Chitra, 2018)	4, 7, 30, 60 d

4.3 Preparation of blood sample for genotoxic analysis

After every exposure period, 10 fish were caught from each group using a small dip net with the least disturbance, and blood was collected from the caudal vein using a heparinized microsyringe. The collected fresh blood was transferred into microcentrifuge tubes for the analysis of genotoxicity, and the analysis were carried out irrespective of the sex of animals.

4.3.1 Micronucleus assay

The blood smear was prepared for the micronucleus test according to the standard method (Heddle, 1973; Schmid, 1975). Frequencies of cellular anomalies were classified into cytoplasmic and nuclear abnormalities (Carrasco *et al*., 1990), and cytogenetic damages were identified (Sumi and Chitra, 2019).

4.3.2 Comet assay

The comet assay was performed according to the method as described by Singh *et al*. (1988). Briefly, the glass slides coated with 1% normal melting point agarose (NMA) was air dried at 4 °C. A uniform layer of 10 µl of fish blood mixed with 0.5% low melting point agarose (LMPA) was spread on the pre-coated agarose slide and solidified. The slides were then subjected to cellular lysing in ice-cold lysing solution (pH 10 at 4 °C) for 1 h. After lysis, the slides were kept in ice-cold electrophoresis buffer (pH 13 at 4 °C) for 20 min for denaturation. Then run in electrophoresis (24 V (~0.74 V/cm) and 300 mA) using the same buffer for 30 min. The slides were washed in neutralization

buffer, stained with acridine orange and observed under Epifluorescent inverted microscope (Olympus CKX41) using green filter (Excitation filter BP480-550C).

The images were captured using a C-mount camera (Optika pro5 CCD camera) and analyzed using Open Comet-Image J, version 1.3.1 software. DNA damage was estimated based on the percentage of DNA in the comet tail. The damages in tail DNA were categorized into five grades as damages less than 5%, 5-20%, 20-40%, 40-95%, and more than 95% with grade zero, one, two, three, and four, respectively (Collins, 2004). The total score was calculated as follows:

Total score = (% of cells in grade 0 × 0) + (% of cells in grade 1 ×1) + (% of cells in grade 2 × 2) + (% of cells in grade 3 × 3) + (% of cells in grade 4 × 4)

4.4 Biochemical analysis

The ovary and testis were dissected out from 10 fish per sex of both control and treatment groups by sacrificing the animal. The gonads were washed with normal saline (0.9%) to remove blood clots and debris and 1% tissue homogenates were prepared in respective buffers using a motor-driven glass Teflon homogenizer on crushed ice for 1 min, centrifuged at 800 *g* for 15 min at 4 °C. The gonadal supernatants were collected, stored at −80 °C for the following biochemical analysis:

4.4.1 Glutathione reductase

Glutathione reductase was assayed by the method of Carlberg and Mannervik (1985). The assay mixture contained phosphate buffer (100 mM, pH 7.6), NADPH (200 mM), EDTA (10 mM), glutathione oxidized (20 mM), and 50 μl of enzyme source. The disappearance of NADPH was measured immediately at 340 nm against enzyme blank at 10 sec intervals for 3 min on a Shimadzu UV-Visible Spectrophotometer. The activity of the enzyme was expressed as nmol of NADPH oxidized/ min/mg protein.

4.4.2 Reduced glutathione

The estimation of total reduced glutathione (GSH) was performed according to the method as described by Moron *et al* (1979). The sample mixture was added to 0.2 M phosphate buffer and 0.6 mM di-thio nitro-benzoic acid (DTNB) in 0.2 M phosphate buffer. The absorbance was read at 412 nm against a blank containing trichloroacetic acid instead of the sample. A series of standards treated similarly were used to determine glutathione content, and the amount of total glutathione was expressed as nmol of GSH formed/ mg protein

4.4.3 Hydrogen peroxide generation

Hydrogen peroxide generation was assayed by the method of Pick and Keisari (1981). The assay mixture contained phosphate buffer (50 mM, pH 7.6), horseradish peroxidase (8.5 units/ mL), phenol red (0.28 nM), dextrose (5.5 nM), and 100 μL of enzyme source was incubated at 32°C for 30 min. The reaction was terminated by the

addition of 60 μL of 10N sodium hydroxide. Absorbance was read at 610 nm against enzyme blank on a Shimadzu UV-Visible Spectrophotometer. The quantity of hydrogen peroxide produced was expressed as nmol hydrogen peroxide generated/ mg protein. For the preparation of the standard curve, a known amount of hydrogen peroxide and all the above reagents except enzyme source were used.

4.4.4 Lipid peroxidation

The level of lipid peroxidation was measured by the method of Ohkawa *et al*. (1979). The stock solution contained equal volumes of trichloroacetic acid (15%, w/v) in 0.25 N hydrochloric acid and 2-thiobarbituric acid (0.37%, w/v). One volume of the test sample and two volumes of stock reagent were vortexed, and heated for 15 min in a boiling water bath. After cooling on ice, the precipitate was removed by centrifugation at 1000 *g* for 15 min and absorbance was measured at 532 nm against enzyme blank. The values were expressed as μmol of malondialdehyde formed/ mg protein. A standard curve was prepared with the known amount of malondialdehyde and all the above reagents except the enzyme source.

4.4.5 Protein carbonyl content

Protein carbonyl content was determined by the most common and reliable method as described by Levine *et al* (1990) with a slight modification (Reznick and Packer, 1994). Briefly, the tissue homogenate was incubated with 2,4-dinitrophenylhydrazine (DNPH) for 60 min. The protein was precipitated by adding 20% trichloroacetic

acid and the pellets are centrifuged at 3400 *g* and washed in ethylacetate: ethanol mixture (1:1) to remove excess of DNPH. The final protein pellet was dissolved in 6 M guanidine hydrochloride. The carbonyl content was determined by reading the absorbance at 370 nm in the spectrophotometer against the blank, and the values are expressed in nmol per mg protein.

4.4.6 Estimation of protein

Total protein was estimated according to the method of Lowry *et al*. (1951). The sample was mixed with alkaline copper reagent and allowed to stand for 10 min at room temperature. Folin-Ciocalteau reagent (1 N) was added to each of the tubes, vortexed, and allowed to stand for 20 min at room temperature. Bovine serum albumin was used as the standard, and the optical density was read at 610 nm on a Shimadzu UV-Visible Spectrophotometer.

4.5 Statistical analyses

All data represented in Tables and Figures were expressed in Mean ± Standard Deviation (SD) for ten animals per group, in replicates. The mean differences between the groups were considered to be significant at $P<0.05$ against the control groups, which were shown as asterisks (*). Comparison of mean among the groups was performed using one-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range as a post-hoc test using SPSS 21.0 statistical software. All biochemical analyses were carried out in triplicate to minimize the statistical errors.

5. RESULTS

5.1 Effect of triclosan on the antioxidant defense system in gonads of the fish, *Anabas testudineus*

Exposure of triclosan at 0.009 and 9 µg L^{-1} (environmentally relevant concentrations), and 176.7 µg L^{-1} (sublethal concentration showed a significant ($P<0.05$) decrease in the activity of glutathione reductase in both ovary and testis of the fish, *Anabas testudineus* (Fig. 1). The level of reduced glutathione also showed a significant ($P<0.05$) reduction in duration and concentration-dependent manner than the respective control gonadal tissues (Fig. 2). However, the levels of hydrogen peroxide generation (Fig. 3), lipid peroxidation (Fig. 4), and protein carbonyl content (Fig. 5) were significantly ($P<0.05$) increased in both ovary and testis after triclosan exposure than the corresponding control groups.

5.2 Effect of triclosan on micronucleus test

The peripheral blood obtained from the control groups showed clear cytoplasm and a distinct compact nucleus without any nucleo-cytoplasmic abnormalities (Figs. 6A and 6B). Triclosan exposure caused cytoplasmic and nuclear abnormalities in the peripheral blood of the fish, *A. testudineus*. Cytoplasmic abnormalities include vacuolated and degenerated cytoplasm (Fig. 6C), formation of sticky cells (Fig. 6D), cytoplasmic bridge (Fig. 6E), cells without cytoplasm (Fig. 6F), and granular cytoplasm (Fig. 6G). The nuclear abnormalities observed were irregular and blebbed

nucleus (Fig. 6C), binucleated and serrated nucleus (Fig. 6D), micronucleus (Fig. 6E), and notched nucleus (Fig. 6H). The percentage of nuclear and cytoplasmic abnormalities increased with an increase in time and concentrations (Tables 1 and 2).

5.3 Effect of triclosan on comet assay

The control and vehicle-control groups showed 96% of Grade A comet scores, which was 0.75 and 0.88 of the total scores, respectively (Table 3). In the triclosan-exposed group, at 0.009 µg L^{-1} concentration, there was a significant ($P<0.05$) increase in the grades of DNA damage with an increase in the duration of exposure showing 2.21 to 14.77 of the total scores (Table 3). The grades of DNA damage were observed to increase at 9 µg L^{-1} concentration of triclosan ranging from Grade 0 to Grade 3 with an increase in total score from 2.51 to 17.79 based on the duration of exposure (Table 3). In the sublethal triclosan exposure group, there was a significant ($P<0.05$) increase in the grades of DNA damage than the environmentally relevant concentration groups (0.009 and 9 µg L^{-1} concentrations) with the total score ranging between 2.82 and 18.72 based on an increase in exposure time (Table 3).

The comet parameters such as percent tail DNA and tail length were also scored after triclosan exposure, which showed a significant ($P<0.05$) increase in both the parameters and the severity of DNA damages were time- and concentration-dependent (Table 4). However, control groups showed no significant differences in the percent tail DNA and tail length.

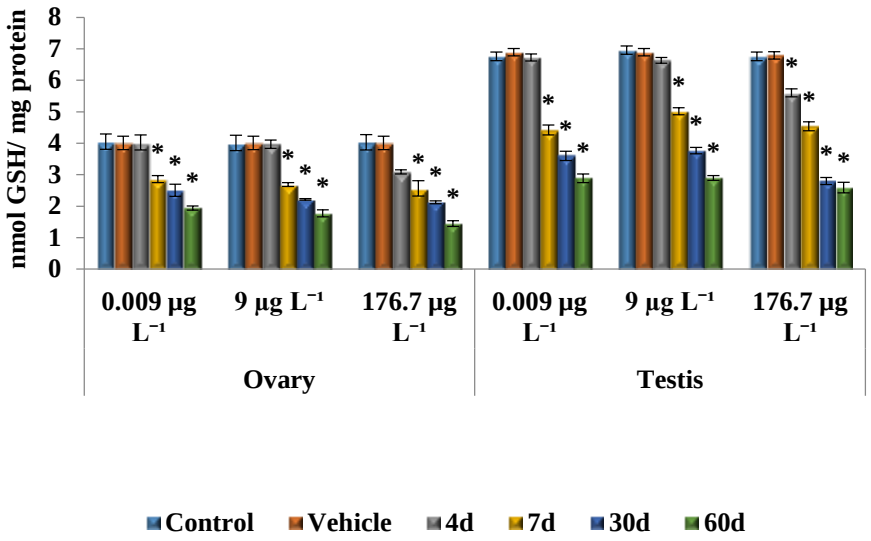

Fig. 1 Effect of triclosan on the activity of glutathione reductase in the gonads of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)

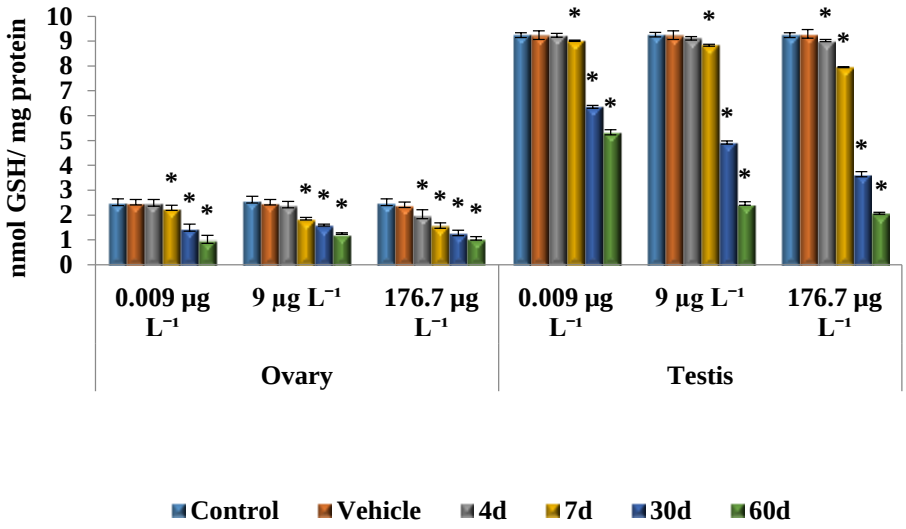

Fig. 2 Effect of triclosan on the level of reduced glutathione in the gonads of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)

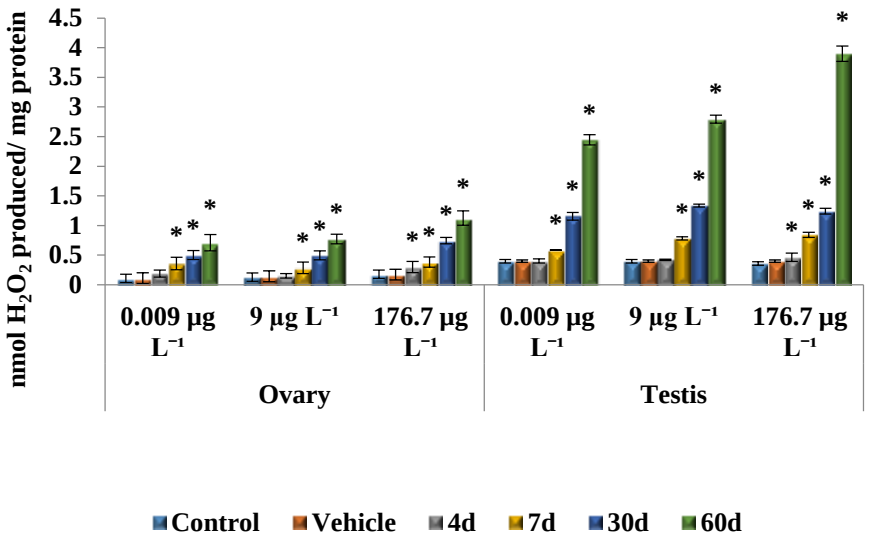

Fig. 3 Effect of triclosan on the level of hydrogen peroxide generation in the gonads of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)

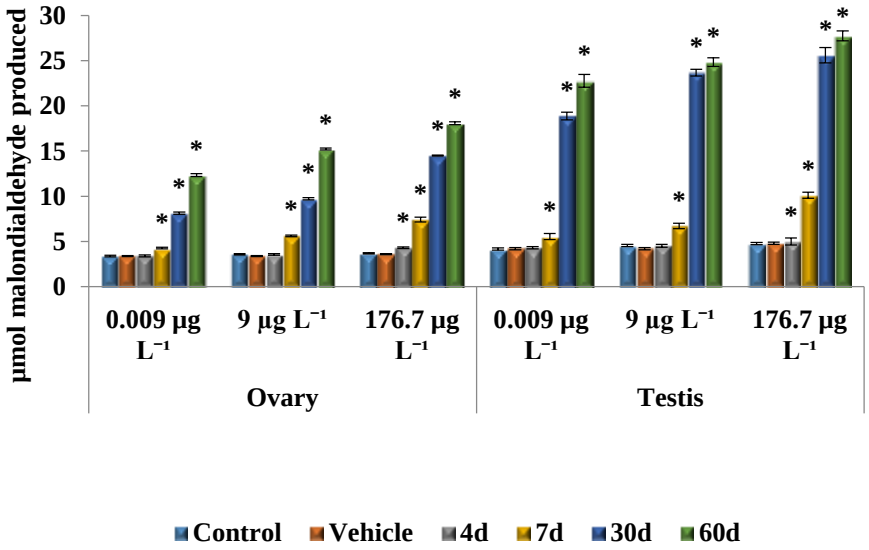

Fig. 4 Effect of triclosan on level of lipid peroxidation in the gonads of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)

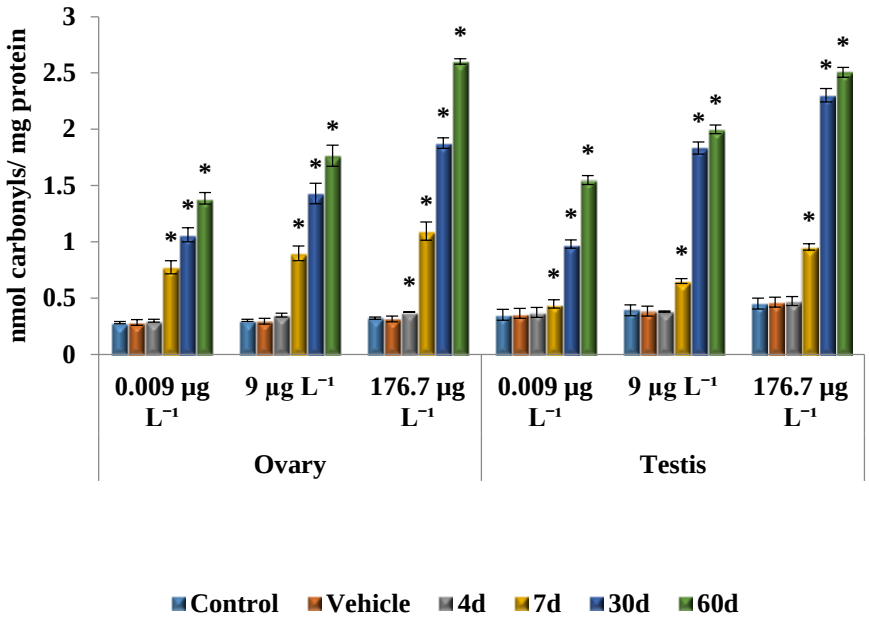

Fig. 5 Effect of triclosan on the level of protein carbonyl content in the gonads of the fish, *Anabas testudineus* (Mean ± SD; n = 10/ group, in replicates; Significant at *P<0.05 against the control groups)

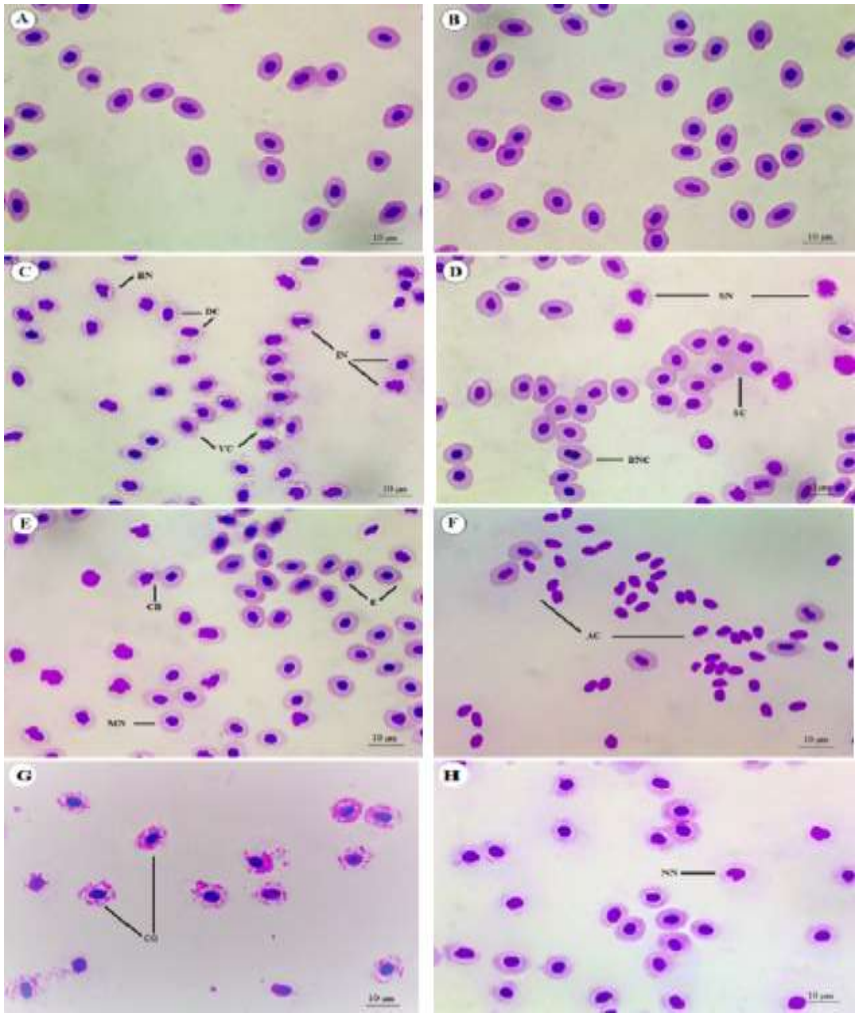

Fig. 6 **Effect of triclosan showing cytonuclear abnormalities in the peripheral blood of the fish, *Anabas testudineus*.** **A**-Control; **B**-Vehicle; **C**-Vacuolated cytoplasm (VC), degenerated cytoplasm (DC), irregular nucleus (IN), blebbed nucleus (BN); **D**-Sticky cells (SC), binucleated cell (BNC), serrated nucleus (SN); **E**-Cytoplasmic bridge (CB), echinocytes (E), micronucleus (MN); **F**-Cells without cytoplasm (AC); **G**-Cytoplasmic granules (CG); **H**-Notched nucleus (NN); **Scale:10 µm**.

Table 1 Effect of triclosan on nuclear abnormalities in the erythrocytes of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at $P < 0.05$ against the control groups; n = 10/ group; Total cells scored-1000 Nos.)

Treatment groups		Blebbed Nucleus	Notched Nucleus	Micronucleus	Irregular Nucleus
Control		1.36±0.26	0	0	1.47±0.15
Vehicle		1.29±0.10	0	0	1.45±0.03
0.009 µg L^{-1}	4 d	3.07±0.064*	1.41±0.13	1.15±0.02	6.54±2.07*
	7 d	4.11±0.364*	3.03±0.33*	1.32±0.21	11.69±2.15*
	30 d	10.20±1.014*	3.49±1.32*	2.18±0.53*	12.18±1.64*
	60 d	12.02±2.34*	5.37±1.73*	3.76±1.26*	21.64±0.12*
9 µg L^{-1}	4 d	3.35±0.06*	1.47±0.06	1.66±0.01*	7.29±0.80*
	7 d	4.74±1.18*	3.08±0.84*	3.92±1.05*	12.41±1.27*
	30 d	11.51±1.95*	4.76±1.51*	10.08±0.90*	15.47±1.53*
	60 d	13.37±0.11*	5.43±1.98*	15.91±1.20*	24.99±0.39*
176.7 µg L^{-1}	4 d	4.511±0.14*	1.68±0.02	1.76±0.01*	12.52±0.21*
	7 d	5.66±0.67*	3.67±1.32*	6.09±1.58*	13.75±1.88*
	30 d	12.34±1.26*	4.63±1.17*	13.06±0.62*	15.72±1.60*
	60 d	14.26±0.85*	5.58±2.42*	18.24±1.85*	27.30±2.61*

Table 2 Effect of triclosan on cytoplasmic abnormalities in the erythrocytes of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at $P < 0.05$ against the control groups; n = 10/ group; Total cells scored-1000 Nos.)

Treatment groups		Sticky cells	Cytoplasmic degeneration	Cytoplasmic vacuoles	Cytoplasmic bridge formation
Control		0.077±0.24	0	0.16±0.36	0
Vehicle		0.064±0.20	0	0.16±0.52	0
0.009 µg L^{-1}	4 d	0.97±2.19	0	0	0.15±0.47*
	7 d	13.23±1.11*	0	9.65±1.68*	3.76±0.90*
	30 d	14.46±2.74*	3.76±0.68*	18.26±3.66*	5.04±1.40*
	60 d	17.26±3.65*	9.43±1.27*	25.50±1.54*	11.61±2.01*
9 µg L^{-1}	4 d	6.63±1.76*	0	1.44±0.26*	3.83±0.80*
	7 d	10.92±1.94*	5.17±0.59*	6.78±2.75*	4.47±1.00*
	30 d	8.16±2.04*	14.64±1.91*	21.87±2.02*	10.14±1.65*
	60 d	11.57±1.74*	25.12±2.30*	15.86±2.36*	4.35±0.95*
176.7 µg L^{-1}	4 d	14.67±1.42*	0	20.74±2.84*	3.74±0.66*
	7 d	32.02±3.07*	13.21±2.01*	25.45±2.37*	6.44±1.27*
	30 d	19.13±1.88*	32.56±1.88*	33.58±0.92*	11.49±0.89*
	60 d	12.86±1.09*	35.27±0.92*	20.46±0.97*	7.79±0.54*

Table 3 Grades of DNA damages after triclosan exposure in erythrocytes of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) indicated significance at P<0.05 against the control groups; n = 10/ group; Total cells scored-100 Nos.)

Treatment groups		Grades of DNA damage					Total score (%)
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	
Control		96.88±0.66	3.13±0.66	0	0	0	0.75
Vehicle		96.33±0.23	3.67±0.23	0	0	0	0.88
0.009 µg L^{-1}	4 d	90.75±0.61*	9.25±0.61*	0	0	0	2.21
	7 d	88.42±0.65*	11.58±0.65*	0	0	0	2.77
	30 d	78.36±0.99*	19.83±0.49*	1.82±0.49*	0	0	5.62
	60 d	60.94±0.36*	24.25±0.20*	7±0.82*	7.81±0.26*	0	14.77
9 µg L^{-1}	4 d	89.5±0.41*	10.5±0.41*	0	0	0	2.51
	7 d	86.25±0.61*	13.75±0.61*	0	0	0	3.29
	30 d	76.52±0.72*	14.59±0.79*	6.45±0.22*	2.44±0.15*	0	8.34
	60 d	57±0.82*	19.59±0.48*	15.47±0.38*	7.94±0.05*	0	17.79
176.7 µg L^{-1}	4 d	88.22±0.48*	11.78±0.48*	0	0	0	2.82
	7 d	83±0.82*	14±1.63*	3±2.45*	0	0	4.79
	30 d	60.65±0.89*	20.75±0.61*	14.94±0.36*	3.66±0.08*	0	14.75
	60 d	54.28±0.69*	23.25±0.20*	12.47±0.48*	10±0.00*	0	18.72

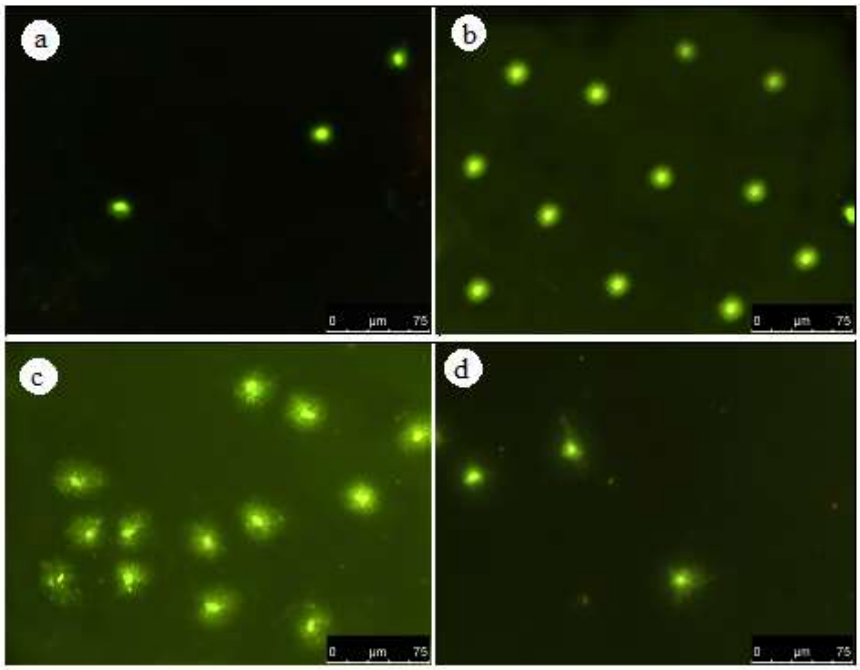

Fig. 7 Representative images of grades of DNA damage in *Anabas testudineus* (a) Grade 0-control and vehicle-control cells; (b) Grade 1; (c) Grade 2; (d) Grade 3

Table 4 Effect of triclosan on the percent tail DNA, and tail length in erythrocytes of the fish, *Anabas testudineus* (Mean ± SD; asterisks (*) denotes significance at P<0.05 against the control groups; n = 10/ group)

Experiment groups		Tail DNA (%)	Tail length
Control		2.73±0.60	0.43±0.30
Vehicle		2.74±0.77	0.43±0.34
0.009 µg L^{-1}	**4 d**	3.02±0.84	0.69±0.35
	7 d	4.08±1.73	3.57±1.36*
	30 d	7.39±1.64*	11.96±2.21*
	60 d	18.84±3.59*	29.14±3.58*
9 µg L^{-1}	**4 d**	3.51±0.61	0.93±0.39
	7 d	4.27±1.35	3.93±1.83*
	30 d	8.58±2.49*	13.87±1.50*
	60 d	24.43±2.84*	30.18±2.67*
176.7 µg L^{-1}	**4 d**	3.84±0.75	1.00±0.34
	7 d	7.00±1.94*	4.75±2.19*
	30 d	16.25±2.27*	14.69±0.58*
	60 d	28.39±3.38*	39.67±3.68*

6. DISCUSSION

6.1 Effect of triclosan on the antioxidant defense system in gonads of the fish, *Anabas testudineus*

Environmental contaminants, including xenoestrogens, are known to imbalance the pro-oxidant and antioxidant defense system leading to the induction of oxidative stress in various aquatic organisms, including fish (Alfhili and Lee, 2019). The overproduction of reactive oxygen species (ROS) subsequently leads to oxidative damage to macromolecules in the cells such as proteins, lipids, and DNA (Patlevic *et al*., 2016). The damages in macromolecules cause several potential adverse effects including inhibition of antioxidant enzyme activity, peroxidative cell membrane damage, DNA lesions, and mutagenesis (Jezek and Hlavata, 2005).

Gonadal tissues of fish are well-equipped with an integrated antioxidant defense system like non-enzymatic and enzymatic antioxidants to defend against ROS production as a consequence of toxicant exposure. The present study evaluated the induction of oxidative stress in gonads of the fish, *Anabas testudineus* after triclosan exposure. The induction of oxidative stress could increase the incidence of oxidative DNA damage thus the study also evaluated triclosan-induced genetic damage in the peripheral blood cells using micronucleus test and comet assay. Some of the valuable antioxidant parameters such as glutathione reductase, reduced glutathione, levels of hydrogen peroxide, lipid peroxidation, and protein carbonyl content were evaluated in the gonads of *A. testudineus*.

The glutathione system constitutes the non-enzymatic antioxidants such as reduced glutathione (GSH), glutathione disulfide (GSSH), and GSH-related enzymatic antioxidants like glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST). The interaction of both the non-enzymatic and enzymatic glutathione systems scavenges the electrons from the free radicals and lipid peroxides thereby alleviate oxidative stress (Szalai *et al*., 2009; Wu *et al*., 2011). Glutathione reductase is a FAD-containing enzyme that catalyzes the reduction of glutathione disulfide (GSSG) to glutathione (GSH) thereby contribute to the redox state of the cells (Couto *et al*., 2016). Glutathione functions as the central redox agent by neutralizing the formation of ROS and 2-oxoaldehyde probably functioning along with the thioredoxin system (Kanzok *et al*., 2001). GSH gets oxidized to GSSG by reacting with the free radicals, or by the catalytic action of glutathione peroxidase or glutathione S-transferase enzymes, whereas glutathione reductase enzyme regenerates GSH through NADPH-dependent reaction (Grant, 2001).

In the present study, fish exposed to triclosan at environmentally relevant concentrations, i.e., 0.009 and 9 µg L^{-1} caused a significant reduction in the activity of glutathione reductase and reduced glutathione content from 7 d onwards. However, sublethal exposure of triclosan, i.e., at 176.7 µg L^{-1} concentration inhibited the activity of glutathione reductase and reduced glutathione content in all treatment groups, in both ovary and testis of the fish illustrating an altered redox state in the gonads. These observations were in accordance with previous findings that triclosan exposure at 50, 100,

and 150 µg L^{-1} concentrations to five-month-old male zebrafish lowered the level of GSH along with a reduction in the activity of GR, which failed to regenerate and restore GSH in the liver tissues (Gyimah *et al*., 2020). Furthermore, another study has revealed that sublethal exposure of triclosan to the early-life stage of zebrafish altered the redox state of glutathione (Falisse *et al*., 2017). The possible mechanism of altered GR and GSH could be due to the down-regulated expressions of antioxidant-related genes as reported in the gill and ovary of *Danio rerio* after triclosan exposure (Wang *et al*., 2020).

The current study observed that triclosan exposure increased the levels of hydrogen peroxide (H_2O_2) generation, lipid peroxidation, and protein carbonyl contents in the gonads of the fish *A. testudineus*. H_2O_2 is not a free radical as it lacks unpaired electrons, but it is more stable and reactive than molecular oxygen, hence it is widely considered as a non-radical reactive substance (Sies, 2014). H_2O_2 can diffuse across the biological membranes, and thus functions as a second messenger molecule regulating several cellular functions such as initiation of cell proliferation, differentiation and migration, maintenance of cell shape and structure, and the recruitment of immune cells (Stone and Yang, 2006). However, high concentrations of H_2O_2 evoke growth arrest causing cell death and other inflammatory responses (Alvarez *et al*., 2016). GSH and GR also play a significant role in detoxifying H_2O_2 and organic hydroperoxides as a second-line of antioxidant defense mechanism (Halliwell and Gutteridge, 1999). The elevated level of H_2O_2 in gonads of the fish after triclosan

exposure indicated the failure of the antioxidant enzyme system to eliminate the oxygen-derived free radical into non-toxic compounds. Another study has reported that phenol exposure induced the generation of H_2O_2 in serum, liver, and ovary of the fish, *Cyprinus carpio* thereby causing oxidative stress (Das *et al*., 2016). Likewise, exposure to fullerene C_{60} nanomaterial has been shown to increase the level of H_2O_2 generation leading to an oxidative imbalance in the gonads of the fish, *Anabas testudineus* (Sumi and Chitra, 2019a).

Cell membranes formed of the lipid bilayer are more susceptible to oxidative stress as the polyunsaturated fatty acids (PUFA) in the membrane degrade to small, more reactive particles such as conjugated dienes, lipid hydroperoxides, and thiobarbituric acid-reactive substances (Halliwell and Gutteridge, 1999). The products of lipid peroxidation such as acrolein, 4-oxo-2-nonenal, 4-hydroxynonenal, and malondialdehyde exhibit free radical activity, and enhance membrane damage associated with several pathologies (Zieba *et al*., 2000). It is well-known that about 40% of gonadal membrane lipids of fish are rich in PUFA, which forms a critical risk factor for peroxidative attack (Ebeid *et al*., 2007). In the present study, malondialdehyde, the advanced oxidation product of lipid peroxidation, was measured as an oxidative stress biomarker in the gonadal tissues to evaluate the triclosan-induced stress. Triclosan exposure at all concentrations increased the level of lipid peroxidation in both ovary and testis of the fish suggesting that the lipophilic nature of the toxicant probably evoked free radical attack on the membrane lipids of gonadal tissue. Further, the increased intracellular ROS could have

failed to incorporate to GSH, and the insufficient enzymatic activity promoted membrane damages leading to enhanced lipid peroxides (Hemalatha *et al*., 2019b). The present results were in agreement with the findings of Gallego *et al*. (2021), where they reported an increase in the level of lipid peroxidation after ibuprofen and triclosan exposure at 25 and 50 µg L^{-1} concentrations in striped catfish *Pseudoplatystoma magdaleniatum*. Dar *et al* (2020) has reported a similar increase in the level of lipid peroxidation in embryos of four food fishes namely *Cyprinus carpio*, *Ctenopharyngodon idella*, *Labeo rohita*, and *Cirrhinus mrigala* after exposure to different sublethal concentrations of triclosan. In another study, exposure to triclosan alone or in combination with different polymers of microplastics elevated the level of lipid peroxidation in the liver of zebrafish (Sheng *et al*., 2021).

ROS generation has been found associated with an irreversible protein modification and degradation, formed by the two most reactive products of lipid peroxidation namely 4-oxo-2-nonenal and 4-hydroxynonenal, which diffuses from the cell membrane into the cytoplasm and nucleus, and react with a variety of proteins and nucleic acids (Dean *et al*., 1997; Dalle-Donne *et al*., 2003). In the present study, protein carbonyls were measured from the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product derived from 2,4-dinitrophenylhydrazine (DNPH) and was used as a biomarker for protein oxidation elicited by ROS production (Kolgiri and Patil, 2017). In the current study, triclosan exposure caused an increase in the level of protein carbonyl content in the gonads of the fish *A. testudineus* thus indicated oxidative modifications of protein. In another study,

sublethal concentrations of triclosan increased the levels of carbonyl protein in the brain of adult zebrafish (Gyimah *et al*., 2020). On the contrary, zebrafish embryos exposed to environmental concentrations of triclosan did not cause any alteration in the protein carbonyl content (Parenti *et al*., 2019).

6.2 Effect of triclosan on micronucleus formation

One of the possible consequences of free radical formation is genetic damage, in which peripheral blood is widely used as a sensitive indicator of genotoxicity (Garcia-Medina *et al*., 2017). The current study monitored the genotoxicity of triclosan using the micronucleus test and comet assay as biomarkers of DNA damage. Micronucleus is small, round dark structures of the whole or fragments of chromosomes that failed to incorporate into the nucleus of daughter cells during cell division (Bolognesi and Hayashi, 2011). The test for micronucleus formation detects clastogens or aneugens, which are involved in the chromosomal breakage and rearrangement, or induction of aneuploidy or an abnormal number of chromosomes, respectively (Oliveira *et al*., 2009; Obiakor *et al*., 2014). On the other hand, the comet assay that detects single-strand DNA breaks, alkali labile sites, and cross-linking are widely used in biomonitoring the environmental stressors in the aquatic environment (Tice *et al*., 2000; Bolognesi and Cirillo, 2014).

The present study used peripheral blood of the fish to evaluate the micronucleus test and comet assay since it avoids the complex cell preparation, and is also able to obtain repeat samples from the same

animal. The results obtained showed several nucleo-cytoplasmic abnormalities in the peripheral blood of the fish after triclosan exposure, which includes the formation of micronucleus, and other nuclear aberrations like irregular, binucleated, serrated, and notched nuclei. Besides, the cytoplasmic abnormalities such as vacuolated and degenerated cytoplasm, formation of sticky cells, cytoplasmic bridges, and cytoplasmic granules along with the cells without cytoplasm were more prominent among the treatment groups. Echinocytes, the spiculated erythrocytes with small and numerous cytoplasmic projections were also noted after triclosan exposure as a result of the altered membrane permeability or impaired metabolic functions, which could be caused due to the overproduction of ROS (Hale *et al*., 2011). Micronucleus formation and other nuclear abnormalities have been reported in the liver cells of goldfish, *Carassius auratus* (Wang *et al*., 2018), in gill and ovary of zebrafish (Wang *et al*., 2020), in the peripheral erythrocytes of *Oreochromis niloticus* (Vijitha *et al*., 2017), *Pangasianodon hypothalamus* (Paul *et al*., 2019), and *Clarias gariepinus* (Jimoh and Sogbanmu, 2021) after triclosan exposure. The cytoplasmic anomalies observed in the current study coincided with other findings on fullerene C_{60} nanomaterial exposure in the erythrocytes of the fish, *A. testudineus* (Sumi and Chitra, 2019b), and in *Oreochromis mossambicus* exposed to silicon dioxide, titanium dioxide, aluminum oxide, and iron oxide nanoparticles (Vidya and Chitra, 2018).

6.3 Effect of triclosan on comet assay

The primary genetic damage induced by triclosan was also evaluated by comet assay or single cell gel electrophoresis assay using specific parameters such as percent tail DNA and tail length in erythrocytes of the fish. The amount of DNA that leaves the nucleus was known by scoring the percent tail DNA, which denotes the actual percentage of DNA damage in a particular cell, while the tail length or DNA migration represents the extent of genetic damage (Kumaravel *et al*., 2009). Exposure of triclosan at all concentrations for long-term duration showed grade 3 DNA damage, and it was noted that the intensity of the damage increased with time and concentration. A study conducted on zebrafish embryos revealed that triclosan exposure caused slight primary genotoxic effects, only at high concentrations (Parenti *et al*., 2019).

Genetic damage scored using percent tail DNA has been found associated with the generation of ROS in erythrocytes of the Indian major carp *Labeo rohita* (Hemalatha *et al*., 2019a), and in *Oncorhynchus mykiss* (Capkin *et al*., 2017). The tail moment and tail length scored in the hepatocytes of the goldfish, *Carassius auratus*, and adult zebrafish showed significant DNA damage only after high concentrations of triclosan exposure (Wang *et al*., 2018; Gyimah *et al*., 2020). The overall results of antioxidant and genotoxic parameters suggested that triclosan-induced oxidative stress thereby contributed to DNA damage in the fish, *Anabas testudineus*.

7. CONCLUSIONS

1. Exposure of triclosan to the fish *Anabas testudineus* at environmentally relevant and sublethal concentrations caused altered redox state in the gonads as evident by the reduction of glutathione reductase enzyme and reduced glutathione content.
2. The elevated hydrogen peroxide level in the gonadal tissues after triclosan exposure indicated the failure of antioxidant enzymes to eliminate free radicals.
3. The increased lipid peroxidation suggested the lipophilic nature of the toxicant that probably evoked the free radical attack on the gonadal membrane lipids.
4. Triclosan exposure caused an increase in the level of protein carbonyl content in the gonads of the fish as indicated by oxidative modifications of protein.
5. The formation of micronucleus, and other nuclear aberrations like irregular, binucleated, serrated, and notched nuclei in the peripheral blood of the fish indicated genetic damage.
6. The cytoplasmic abnormalities were represented as vacuolated and degenerated cytoplasm, formation of sticky cells, cytoplasmic bridges, and cytoplasmic granules.
7. The comet tail DNA and tail length scored in the erythrocytes of the fish after triclosan exposure confirmed genetic damage.
8. Altogether, the study demonstrated that triclosan exposure induced oxidative stress and genotoxicity in the fish, *A. testudineus*.

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CHAPTER 7

General Conclusions

Chapter 7 General Conclusions

CONTENTS

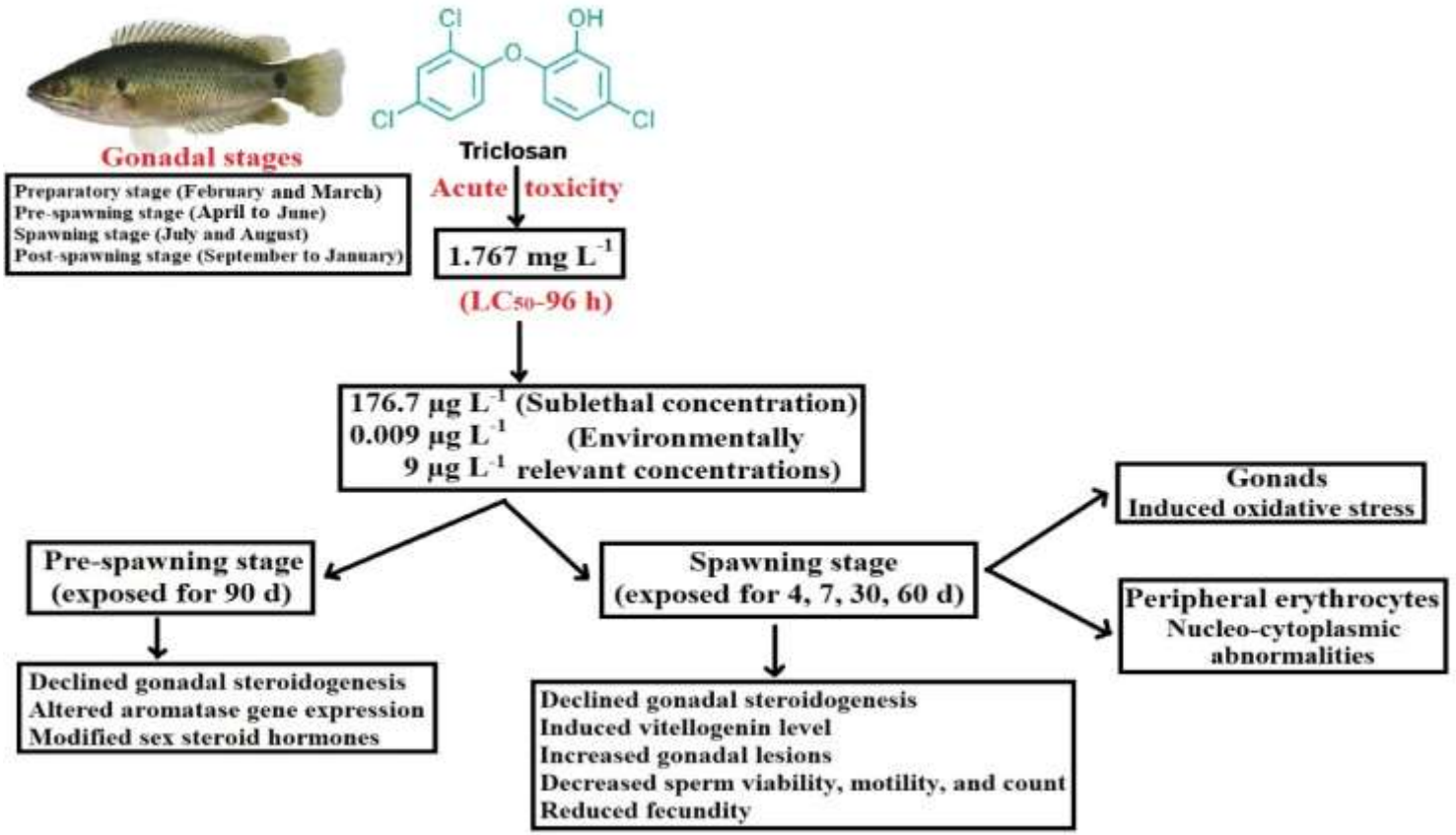
GENERAL CONCLUSIONS

The research study detailed under the topic "Evaluation of triclosan-induced reproductive toxicity in the freshwater fish, *Anabas testudineus* (Bloch, 1792)" was summarized in six different chapters. The major findings acquired out of each chapter are listed below:

- ✓ In Chapter 1, a general introduction was elaborated on the environmental contaminants with special emphasis on triclosan, one of the pharmaceutical and personal care products, its physico-chemical properties, toxicokinetics, and fate on aquatic organisms, particularly on fish species.
- ✓ In Chapter 2, four different stages of gonadal development in the fish under the laboratory conditions were determined as preparatory stage from February to March, pre-spawning stage from April to June, spawning stage from July to August, and post-spawning stage from September to January, showing the fecundity rate between 6,500 and 11,000 eggs.
- ✓ In Chapter 3, the median lethal concentration of triclosan was examined in the freshwater fish, *Anabas testudineus* using probit analysis, which was 1.767 mg L^{-1} concentration.
- ✓ In Chapter 4, the impaired gonadal development in the fish, *A. testudineus* was identified during the pre-spawning period as evident by declined gonadal steroidogenesis, altered aromatase gene expression, modified levels of serum gonadotropins and sex hormones.

- ✓ In Chapter 5, it was concluded that exposure to triclosan during the spawning period altered the biosynthesis of sex steroid hormones by declining the activities of steroidogenic enzymes. Besides, induction of vitellogenin, gonadal lesions, and reduction in sperm motility, viability, and count of male fish along with a decline in the fecundity of female fish indicated that triclosan affected the normal physiology of reproduction in the fish, *A. testudineus.*
- ✓ In Chapter 6, the induction of oxidative stress in gonads, and genotoxicity in peripheral erythrocytes were demonstrated after triclosan exposure in the fish *A. testudineus.*

Collectively, these observations provide important implications for understanding the reproductive physiology of the fish, *A. testudineus*. The study also addressed the adverse effects of triclosan by analyzing several reproductive endpoints such as gonadosomatic index, vitellogenin level, aromatase gene expression, serum hormone levels, fecundity, sperm indices, and gonadal histology, which suggested reproductive toxicity of the compound.


Gonadal stages
Preparatory stage (February and March)
Pre-spawning stage (April to June)
Spawning stage (July and August)
Post-spawning stage (September to January)
Triclosan
Acute toxicity
1.767 mg L-1
(LC50-96 h)
176.7 µg L-1 (Sublethal concentration)
0.009 µg L-1 (Environmentally
9 µg L-1 relevant concentrations)
Pre-spawning stage
(exposed for 90 d)
Declined gonadal steroidogenesis
Altered aromatase gene expression
Modified sex steroid hormones
Spawning stage
(exposed for 4, 7, 30, 60 d)
Declined gonadal steroidogenesis
Induced vitellogenin level
Increased gonadal lesions
Decreased sperm viability, motility, and count
Reduced fecundity
Gonads
Induced oxidative stress
Peripheral erythrocytes
Nucleo-cytoplasmic
abnormalities

RECOMMENDATIONS FOR FUTURE INVESTIGATIONS

Many studies have clearly illustrated the impacts of triclosan in a wide variety of fish species. The estrogenic and anti-androgenic effects of triclosan have been well-documented in the present study. However, the studies on multi- and transgenerational effects of triclosan in fish were not clearly elucidated. The extension of multi- and transgenerational studies will help to elucidate the underlying mechanism of toxicity and distinguishes the direct and indirect (maternal) effects of triclosan on the germlines. The mode of action of triclosan can be interpreted only through the multiple pathways including developmental, reproductive, metabolic, and endocrine functions through the activation of estrogen, androgen or thyroid hormone receptors. In this context, the extension of studies using molecular mechanisms may shed light on this aspect.

Appendix

LIST OF PUBLICATIONS

- **C.V. Priyatha** and K. C. Chitra (2018). Acute toxicity of triclosan on the native freshwater fish, *Anabas testudineus* (Bloch, 1792): behavioural alterations and histopathological lesions. International Journal of Life Sciences. Print ISSN: 2320-7817; Online ISSN: 2320-964X. 6(1): 166-172.

- V.S. Amaya, **C.V. Priyatha** and K. C. Chitra (2018). Haematological responses in the freshwater fish, *Anabas testudineus* (Bloch, 1792) exposed to sublethal concentration of acid orange 7. Journal of Global Biosciences (ISSN 2320-1355). 7(8): 5536-5549.

- P. Shinjina Fathima, **C.V. Priyatha** and K. C. Chitra (2018). Ameliorating effect of vitamin C on oxidative stress induced by acid orange 7 in the gill of the fish, *Anabas testudineus* (Bloch, 1792). Research and Reviews: A Journal of Toxicology. ISSN: 2231-3834 (Online), ISSN: 2349-1264 (Print). 8(3): 15-27.

- **C.V Priyatha** and K. C. Chitra (2019). Sublethal toxicity of acid orange 7 in the freshwater fish, *Anabas testudineus* (Bloch, 1792), and the role of vitamin C as antioxidant in the prevention of oxidative stress. International Journal of Scientific Research in Biological Sciences. UGC No. 63784. ISSN: 2347-7520 (O). 6(2): 92-101. https://doi.org/10.26438/ijsrbs/v6i2.92101.

- A. Rishin, **C.V. Priyatha** and K. C. Chitra (2019). Induction of genetic damage in peripheral erythrocytes of the fish, *Anabas testudineus* (Bloch, 1792) exposed to sublethal concentration of acid orange 7. Research and Reviews: A Journal of Life Sciences. ISSN: 2249-8656 (Online), ISSN: 2348-9545 (Print); 9(2): 83-96.

- Fathima Shincy, Sreya Asok, T. Sreelakshmi, **C.V. Priyatha** and K.C. Chitra (2020). Toxic effects of an organophosphorus pesticide, malathion (50% EC) on the freshwater fish, *Oreochromis mossambicus* (Peters, 1852). International Journal of Fisheries and Aquatic Studies. ISSN: 2347-5129 (E); 2394-0506 (P) 8(2): 57-60.

- N. Vijayakumar, K. Amritha, **C.V. Priyatha** and K. C. Chitra (2020). Sodium benzoate induced reproductive and metabolic changes in *Anabas testudineus* (Bloch, 1792). Research and Reviews: A Journal of Life Sciences. ISSN: 2249-8656 (Online), ISSN: 2348-9545 (Print); 10(1): 56-71.

- P. K. Aswathy, **C.V. Priyatha**, Nikhil John and K. C. Chitra (2021). Triclosan at environmental concentration alters the hepatic antioxidant defense system in the fish, *Anabas testudineus* (Bloch, 1792). Aquaculture Research. ISSN:1365-2109 (John Wiley & Sons). doi:10.1111/are.15534.

- **C.V. Priyatha** and K. C. Chitra (2021). Assessment of triclosan-induced oxidative stress and genotoxicity as biomarkers in the fish Anabas testudineus. Under review in Drug and Chemical Toxicology.

- **C.V. Priyatha** and K. C. Chitra (2021). Influence of triclosan on the reproductive physiology of *Anabas testudineus* during the spawning phase. Under review in Journal of Drug and Chemical Toxicology.

- **C.V. Priyatha** and K. C. Chitra (2021). Chronic effect of Triclosan on the gonadal development in the pre-spawning phase of the *Anabas testudineus*. Under review in Ecotoxicology.

- **C.V. Priyatha** and K. C. Chitra (2021). Molecular identification of *Anabas testudineus* and assessment of the gonadal development under controlled condition. Under review in Journal of Fisheries.