STUDY OF ECOTOXICOLOGICAL IMPACTS OF ANTIBIOTICS AND ITS REMOVAL USING PLANT SOURCES

A Thesis

Submitted in partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

In

ZOOLOGY

By

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LOVELY PROFESSIONAL UNIVERSITY

PUNJAB

2021

Declaration

I hereby declare that the thesis entitled, **"Study of ecotoxicological impacts of antibiotics and its removal using plant sources"** submitted for the doctor of philosophy in zoology is entirely my original work and all ideas and references have been duly acknowledged. It does not contain any work for the award of any other degree or diploma at any university.

Investigator

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Certificate

This is to certify that Mr. Aijaz Ahmad Mala, has completed the thesis entitled "**Study of ecotoxicological impacts of antibiotics and its removal using plant sources"** under my guidance and supervision. To the best of my knowledge the present work is the result of his original investigation and study. No part of the thesis has ever been submitted for any other degree or diploma at any university.

The thesis is fit for the submission and the partial fulfillment of the condition for the award of **DOCTOR OF PHILOSOPHY IN ZOOLOGY.**

Signature of Advisor:

Date: **Dr. Joydeep Dutta**

Abstract

Antibiotics are considered as one of the amazing discoveries which are extensively in use for the treatment of disease. However, due to continuous discharge and persistence in the aquatic environment, antibiotics are detected almost in every environmental matrix. These molecules or their metabolites are discharged into the aquatic environment and contineous exposure results in emergence of resistance among microorganism. Recent reports have suggested that bacteria have gained resistance even to common antimicrobials. Their potential toxicity on the non target organisms are considered as a serious environmental apprehension. Antibiotics are known as emergent pollutants because of their toxicological properties. Therefore, their presence in the aquatic environment have gained significant research interest. The present research work was carried out to test the toxic effects of cephalosporin (cefixime, cefotaxime) and fluoroquinolone (gemifloxacin and gatifloxacin) antibiotics on model organism *Daphnia magna* and *Danio rerio*.

In order to evaluate the biological risk of antibiotics, the toxicity testing was carried out on *D. magna.* Based on range finding test, the test concentration used in the acute test was as: 0, 2.5, 8.0, 25, 80 and 250 mg/L for cefixime and cefotaxime antibiotics and 12.34, 37.03, 111.11, 333.3 and 1000 mg/L for gemifloxacin and gatifloxacin antibiotics. A total of 5 *D. magna* neonates (24 hrs) were treated per concentration. The immobility of *D. magna* was recorded after 48 hrs, and neonates which shows no response within 15s gentle agitation were considered as immobile. The concentration of antibiotics that cause 50% immobility (48h-EC_{50}) was calculated. The test guidelines provided by OECD, was followed for chronic testing and based on 48h-EC_{50} from acute testing, exposure concentrations were selected. The test animals were exposed to cefixime (0, 0.70, 2.1, 6.3, 18.19, and 56.7 mg/L) and cefotaxime (0, 0.20, 0.60, 1.8, 5.4 and 16.2 mg/L), gemifloxacin (0, 3.5, 10.5, 31.5, 94.5, 283.5 mg/L) and gatifloxacin (0, 2.5, 7.5, 22.5, 67.5, 202.5 mg/L) antibiotics by using ten neonate individuals as replicates during 21 days. The endpoints analysed along the test were the survival of neonates, first day of brood, young ones per brood/female.

The 48h-EC_{50} value observed for cephalosporins i.e. cefixime was 77.92 mg/L and for cefotaxime was 25.82 mg/L respectively against *D. magna*. For fluoroquinolone

antibiotics the EC_{50} value for gatifloxacin was 330.8 mg/L and for gemifloxacin was 489.42 mg/L respectively. In chronic toxicity testing survival of *D. magna* was not significantly inhibited by cefixime antibiotic when exposed upto 2.1 mg/L and survival was observed to be 85 % at 56.7 mg/L. No significant difference was observed in the first day of brood and control. The number of young ones per brood and young ones per female were not affected with the increase in cefixime antibiotic concentration. On treating with cefotaxime antibiotic, the survival of daphnids significantly reduce with the increase in antibiotic concentration and 50% daphnids survived at the highest concentration (16.2 mg/L). Delay in reproduction at concentrations higher than 5.4 mg/L was observed. The number of young per female were significantly decreasing after 0.60 mg/L antibiotic concentration and number of young ones per brood were also significantly decreasing after 1.8 mg/L cefotaxime concentration in the chronic assay. In case of gemifloxacin the first day of reproduction had little effect till 10.5 mg/L and after 31.5 mg/L the toxic effect of the antibiotic concentration could not be tested since all daphnids die at these concentrations. The average number of neonates per female was constantly decreasing with the increase in concentration when compared with control. The number of young ones per brood decreased at 10.5 mg/L and after 31.5 mg/L no survival of neonates were recorded. With the increase in gatifloxacin concentration survival of daphnia was decreasing and at 202.5 mg/L, 100% mortality was observed. The initial concentrations of gatifloxacin had little effect at first day of reproduction and after 67.5 mg/L the toxic effect of the antibiotic concentration could not be tested since all daphnids die at these concentrations. Comparing with control the average number of young per female were also affected and at highest concentrations (67.5 and 202.5 mg/L) and no production of young ones were observed. The number of young per brood significantly decreased at 22.5 mg/L and no daphnid was observed after 67.5 mg/L gatifloxacin concentration.

In our study zebra fish, which are considered as a crucial link between environmental pollution and human health were also exposed to cephalosporin (cefixime, cefotaxime) and fluoroquinolone (gemifloxacin, gatifloxacin) antibiotics. Newly fertilized zebra fish embryos were exposed to the antibiotics for a period of 96 hrs. The antibiotic concentrations selected were 100, 25, 12.5, 6.25 and 3.125 µg/ml.

Every after 24 hrs, developmental parameters were recorded which includes: lack of somite formation, coagulated embryos, mortality percentage and non-detachment of tail. Any positive outcome in one of these observations mean that the zebra fish embryos are dead. The survival of embryos were observed 100% in all concentrations of cefixime, cefotaxime, gemifloxacin and gatifloxacin antibiotics. 0% mortality was observed in negative control and internal plate control. There were no lethal effects and other malformations recorded in embryo development. The exposure of embryos to antibiotic concentrations up to 100 µg/ml did not cause any mortality within 96 hrs of exposure and they were found to be safe under the tested experimental conditions in the present study. From the above studies antibiotics were found quite toxic to invertebrate (*Daphnia magna*) forms and the persistence of antibiotics in aquatic environment has raised problems and therefore their removal from water bodies is of must importance.

We also carried out adsorption experiments to remove antibiotics from synthetic water by using beads as adsorbent material. The beads were prepared by mixing Chitosan (C), Walnut (W) and Almond (A) shell powder (in combination- AWC 2:1:1, CAW 2:1:1, WAC 2:1:1). Beads prepared were characterized by Fourier Transform Infrared Spectroscopy analysis (FTIR), Energy Dispersive X-ray analysis (EDX) and Scanning Electron Microscope (SEM). The parameters like contact time (30-180 min), initial concentration (30–50 mg/L), pH (3–11) and adsorbent dose (0.1–1.0 g) were systematically investigated. The experimental data was fitted with Freundlich and Langmuir isotherm models and pseudo-first-order and pseudo-second order kinetic models. The FTIR analysis confirm the presence of N-H, O-H and C-O groups, which are considered as a good choice for the adsorption process. The Scanning electron micrographs confirm the presence of sparsely scattered fissures and occasional pores which can be helpful in the adsorption of antibiotics. The Energy dispersive spectra (EDX) define the elemental peaks of ACW, CAW and WAC beads. The elemental composition of beads confirm the presence of carbon, oxygen and in minor amount nitrogen also. When the adsorption dosage was varied from 0.1 to 1.0 g. The highest percentage removal of cefixime antibiotic on AWC beads was 89%, on CAW beads was 88% and on WAC beads was 90% respectively. The cefotaxime antibiotic adsorption on AWC beads was 88%, on WAC beads 90.5% and on CAW

beads 85.7% respectively. The highest gemifloxacin adsorption was 89% on AWC beads, 90% on CAW beads and 92% on WAC beads respectively. Adsorption of gatifloxacin increased from 81% to 88% on AWC beads, 80% to 88% on CAW beads and 81% to 88% on WAC beads. With the increase in time period from 30-180 minutes, removal percentage of antibiotics were also increasing. The maximum adsorption efficiency of cefixime antibiotic on AWC beads was 81%, on CAW beads 80.69% and on WAC beads 84.66% respectively after 180 min. The adsorption percentage of cefotaxime antibiotic on AWC beads was 84.0%, on CAW beads 86.3% and on WAC beads 85.0% respectively. While in case of gemifloxacin antibiotic adsorption percentage reached 86%, 90% and 85% in case of AWC, CAW and WAC beads respectively in 180 min. The highest percentage removal of gatifloxacin on AWC, CAW and WAC beads were 84%, 88% and 88% respectively. The optimum adsorption efficiency of cefixime antibiotic on AWC (79%) and CAW (81%) beads were observed at pH 7.0. Whereas on WAC (82%) beads was observed at pH 5.0. Optimum adsorption of antibiotic was observed at pH 5.0, with cefotaxime removal rate of about 83.42% on AWC, 84.58% on CAW and 84.80% on WAC beads respectively. The maximum gemifloxacin removal was observed at pH 5.0 on AWC beads (82.0%) and at pH 7.0 on CAW (84%) and WAC (80%) beads respectively. The adsorption of gatifloxacin on CAW (85%) beads was observed at pH 5.0 and at pH 7.0 on AWC (84%) and WAC (82 %) beads respectively. With the increase in initial concentration of antibiotics from 10 to 50 mg/L, the adsorption was observed to be decreasing on all the three types of beads. The adsorption of cefixime antibiotic decreased from 93 to 60% in case of AWC and WAC beads respectively and 94 to 65% in case of CAW beads. The cefotaxime antibiotic removal percentage was decreased from 94% to 68%, 83% to 56%, and 85% to 49% on WAC, AWC and CAW beads respectively. The removal of the gemifloxacin decreased from 95% to 65 % on AWC beads, 93 % to 68 % on CAW beads and 94% to 68% on WAC beads respectively. The adsorption of gatifloxacin on AWC beads decrease from 96% to 63% on AWC beads, 96% to 65% on CAW beads and 94% to 62% on WAC beads respectively. The experimental data for cefixime antibiotic fitted best with Langmuir model for AWC and CAW beads and Freundlich model for WAC beads. For cefotaxime antibiotic the experimental data fitted best with Langmuir model for WAC beads and Freundlich model for CAW beads. Whereas, ACW beads follows both

Langmuir and Freundlich isotherm models. In case of gemifloxacin antibiotic Langmuir model is better followed in case of CAW beads and Freundlich adsorption better in case of AWC and WAC beads. For gatifloxacin, Langmuir model is better followed in case of ACW beads and Freundlich adsorption better in case of CAW and WAC beads. The adsorption was observed to follow Lagergren pseudo second order better than Lagergren first order kinetic model on all types of beads.

In our study we highlighted the ecotoxicological effects of cephalosporin and fluroquinolone antibiotics on *Daphnia magna* and *Danio rerio*. The result suggested that antibiotics induce reproductive toxicity in *D. magna* and showed no effect on *D. rerio*. Our findings also demonstrated the reference value for the preparation of efficient plant waste material beads.

Acknowledgements

Thanks to **The Merciful Almighty Allah** for all the countless gifts you have offered me.

I wish to express my sincere thanks to my guide, Dr. Joydeep Dutta, Professor and Head of Department, Department of Zoology and Botany, School of Bioengineering and Biosciences, Lovely Professional University Phagwara (Punjab) for his moral support all along during my project work.

I would like to thanks Prof. Neeta Raj Sharma, Head, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara for her expert facilities provided during the study.

I would like to express my thanks to Dr. Amit Sehgal Assistant Professor, Department of Zoology, Lovely Professional University Phagwara, Punjab, and Dr. Rahul Singh Assistant Professor, Department of Zoology, Lovely Professional University Phagwara, Punjab and Er. Chirag Chopra, Assistant Professor and Head of Lab, Department of Molecular Biology and Genetic engineering, Lovely Professional University Phagwara, Punjab, who have helped me the most throughout my work.

I am thankful to Mr. Aman sir, Lab-technician, for his cooperation and encouragement all during my lab work. It was always a pleasure to think about the nice environment, clean glassware and guidance provided in the lab.

It is a great pleasure to acknowledge my deepest thanks and gratitude to Dr. Bilques Farooq, for her kind endless help, encouragement, generous advice and support during the study.

I am also grateful for the vital guidance and assistance provided by my precious friend Dr. Sumaya Farooq.

A very special word of thanks goes to Ms. Denita who have always been a major source of support when things would get a bit discouraging.

I am also thankful to Dr. Imtiyaz Ahmad for the most vital encouragement to keep my motivation alive.

Two person at the department of zoology, deserve a special word of gratitude one is Ms Nafiaah and other Ms Sufiara, who have given me valuable support during ma project.

And finally, I owe my thanks to my parents especially (My father Gh. Rasool Mala and my sweet dear mother Khatoon Begum) for being my confidant and for being one of my best teachers. You taught me how to choose battles wisely and how to deal with life. You made me realized how important each day is.

Thank you for valuing me.

Aijaz Ahmad Mala.

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Introduction

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INTRODUCTION

Pharmaceuticals are widely used worldwide for the management of various ailments. They are discharged into the aquatic bodies during various stages of the product lifecycle. These pharmaceuticals are detected in different water sources i.e. wastewater, drinking water, ground water and surface water. These are identified as emerging contaminants as they remain active in the environment even after their usage. The presence of pharmaceuticals or their active compounds in water bodies adversely affects the aquatic environment and poses risk to human health. Based on adverse effects on living organisms, these are included on a dynamic 'watch list' by EU Water Framework Directive (Debarros *et al*., 2018). The contaminants comprise a very diverse spectrum of compounds. These compounds are not regulated by any environmental legislation even though they represent an environmental risk. Therefore, there is concern for their potential health risk as they intend to enter food chain and drinking water facilities.

Among the pharmaceuticals, antibiotics are broadly prescribed for the treatment of infectious diseases in humans, animals and agriculture. Increased usage in the recent years probably states the ultimate demand of antibiotics which is 1×10^5 and 2×10^5 tons (Wang *et al*., 2019). Larger portion of the antibiotics taken by humans and animals subsequently get excreted through urine and faeces. These parent and unmetabolized compounds are of concern as they remain in STPs and in the water bodies. These are considered as "pseudo-persistent".

1.1. Presence of antibiotics in water bodies

The unmetabolized antibiotic from medical and veterinary systems are the main pollutants of the environment. The other sources include STPs of industries, septic leakage and agriculture. About 30% to 90% of antibiotics are excreted as the parent compound used as veterinary medicine (Balakrishna *et al*., 2017). Several investigators have reported the antibiotic presence in different water compartments including WWTP effluents (Guerra *et al*., 2014), sediments (Awad *et al*., 2014), surface water (He *et al*., 2015) and ground water (Ma *et al*., 2015). Different antibiotic classes fluorquinolones (Vo *et al*., 2016), tetracyclines (Ma *et al*., 2015),

sulfonamides (Bai *et al*., 2014) and macrolides (Ma *et al*., 2015) are detected in different aquatic systems. Because of imperfect technological advancement of their removal, these antibiotics have formed their ways into drinking facilities.

1.2. Antibiotics: An Environmental Concern

Antibiotics get access to water bodies from different sources i.e, aquaculture, animal husbandry, chemical manufacturing and wastewater treatment plants. In contrast to human's, waterborne organisms are exposed to contaminants (especially antibiotics) throughout their lifetime. However, not much is known about the long-term hazards exposure of antibiotics at lower concentrations. But reports states that organisms are highly susceptible to the adverse effects of antibiotics, when they are exposed for longer duration.

1.2.1. Toxic effect of waterborne antibiotics

Antibiotics show acute and chronic toxic effects on living organisms. Studies have reported that chronic exposure is critical because of their half-life resulting in the development of toxicity to many microorganisms (Cycon *et al*., 2019). Antibiotics reported to act as potential genotoxic agents which are proved through animal and microbial assays like the SOS chromo test on *E. coli*, point mutation test (Ames test) on *Salmonella* species (Isidori *et al*., 2005), etc. Higher plants also being used as the models to test the genotoxicity of antibiotics through micronucleus formation, root tip chromosomal aberration assay and sister chromatid exchange. These drugs are reported to induce chromosomal aberrations in plants. In total antibiotics, proved to be harmful once they gain access to the water systems and thereby, cause drastic damage to living forms.

Other effects of antibiotics reported are: -

- On growth
- Survival of microflora and fauna
- Productivity loss of aquatic ecosystem
- Potential development of antibiotic-resistant bacteria
- Allergic reactions in humans
- Greenhouse gas emissions

- Reprotoxic
- Oxidative stress
- Cytotoxicity
- Bioluminescence
- Delayed cell growth

Antibiotics in water also effects non target organisms, however reports are poor. The ecological effects are poorly investigated concerning non target bacteria and their related ecological functions.

1.2.2. Development of antibiotic resistance: A major concern

Pathogens exposed to antibiotics for longer period results in spreading resistance among them. Recent reports have suggested that bacteria have gained resistance even to common antimicrobials. Antibiotics were earlier causing nosocomial infections to hospitalize patients but nowadays are spreading and causing severe illness to the community. As per WHO 2014, *E. coli*, *K. pneumoniae* and *S. aureus* are the greatest agents of hospital acquired infections. In a study reported by Arslan *et al*. (2005), the prevalence of infections is increasing due to antibiotic-resistant bacteria which make UTI treatment more difficult. Fluoroquinolones like ciprofloxacin, norfloxacin, ofloxacin, lomefloxacin are the major contributors to developing resistant microorganisms' example *E. coli, Acinetobacter, Brevundimonu*. The other broad spectrum antibiotics causing resistance are sulfonamides and tetracyclines.

The different sources bring in the antibiotics to the aquatic environment wherein the microorganisms slowly develop resistance by producing destructive enzymes, mutation and pumping antimicrobial by effluxing, reducing the permeability to antibiotics and creating bypasses. These resistant bacteria gain through to humans through alternative pathways such as environmental exposure, person to person transfer and direct exposure to animals. Selective pressure and development of resistant genes are two basic factors for the development of antibiotic bacterial resistance. Resistance to antibiotics makes the treatment of infections extremely difficult, costly and in many cases results in high morbidity and mortality.

1.2.3. Mechanism of action of fluoroquinolone and beta lactams

The aim of antibiotics are to target five bacterial points: ribonucleic acid synthesis, protein synthesis, deoxyribonucleic acid (DNA) synthesis, cell wall synthesis and intermediary metabolism. The bacteria shows resistance to antibiotics that attack these targets sites. Understanding of new antimicrobials and their mechanisms of action are vital (Speltini *et al*., 2015). Fluoroquinolone are potent antibacterial agents that target two related enzymes, DNA gyrase and DNA topoisomerase IV. Gyrase is responsible for introducing negative supercoils into DNA and for relieving torsional stress expected to accumulate ahead of transcription and replication complexes. Topoisomerase IV provides a potent decatenating (unlinking) activity. Both gyrase and topoisomerase IV are essential enzymes, and, therefore, agents that attack them are expected to block bacterial growth (Drlica, 1999). The quinolones have as their targets two essential Eukaryotic enzymes, DNA gyrase (topoisomerase II) and DNA topoisomerase IV. These two enzymes act by passing one region of duplex DNA through another and during that process the drugs trap a reaction intermediate containing quinolone, enzyme, and broken DNA. The resulting ternary complexes block DNA replication and for some bacteria death occurs within hours. (Drlica and Malik, 2003). The fluoroquinolones, however, do not simply eliminate enzyme function — they actively poison cells by trapping these two topoisomerases on DNA as drug/enzyme/DNA complexes in which double-strand DNA breaks are held together by protein. (Drlica, 1999). The beta-lactams on the hand inhibit the last step in peptidoglycan synthesis by acylating the transpeptidase involved in cross-linking peptides to form peptidoglycan. The targets for the actions of beta-lactam antibiotics are known as penicillin-binding proteins (PBPs). This binding, in turn, interrupts the terminal transpeptidation process and induces loss of viability and lysis, also through autolytic processes within the bacterial cell. Cephalosporins have same mode of action as other β-lactam antibiotics. They disrupt the synthesis of the peptidoglycan layer forming the eukaryotic cell wall (Eckburg *et al*., 2019).

1.3. Antibiotic removal process

Conventional technologies include filtration, coagulation/ flocculation/ sedimentation, oxidation, fenton and photo fenton, ion exchange, activated sludge, oxidation process, carbon filtering, constructed wetlands, nanotechnology, membrane distillation, reverse osmosis are used for the removal of pollutants. Most of these techniques applied in waste water treatment plant and drinking water treatment plants were not

able to remove antibiotics. Activated carbon have also used as adsorbent materials, which showed good results in antibiotic removal. But the activation of activated carbon requires high cost of production and are not considered as environment friendly. High-cost functioning for the removal of antibiotics demands to look for more appropriate, convenient, cost-effective removal technologies of antibiotics for a safer environmental system. Therefore, a need for efficient adsorption mechanism is in demand to prevent the aquatic organisms from the harmful effect of the leftover antibiotics and their metabolites.

1.3.1. Adsorption technologies

Adsorption is widely acknowledged as an efficient and effective method for water contamination applications for the removal of antibiotics from water bodies, because of its easy operation, simple design and suitability for batch processes. Adsorption technique removes pollutents without producing dangerous secondary metabolites and there are more chances of removing pollutants than other wastewater treatment methods. Some low-cost adsorbents which have already used includes use of sawdust (Bajpai *et al*., 2012), biochars (Zheng *et al*., 2013), chitosan (Vakili *et al*., 2015), cellulose (Rathod *et al*., 2015), palm leaves (El-shafey *et al*., 2012), paper towel (Xie *et al*., 2016), corn bracts (Yu *et al*., 2017). Some other adsorbents used - Clays (Avisar *et al*., 2010), agricultural residues (Ahmed *et al*., 2013), lotus stalk (Liu *et al*., 2011), walnut shell (Yu *et al*., 2016) and rice husk (Chen *et al*., 2016). The adsorbents used in powder form remain suspended in water reducing clarity of water. To overcome this problem, other alternative ways of framing these agricultural wastes in other form like beads would be an advantageous idea for adsorption technology. The beads would not only help in adsorption but also proved beneficial in two ways: One is easy removal from water body due to their size and other will not degrade the water quality by interfering with light.

Chitosan is widely used as sorbents for elimination of wide range of waterborne contaminants including organic and inorganic pollutants viz. Dyes (Chiou *et al*., 2004), Antibiotics (Adriano *et al*., 2005), Metals (Igberase *et al*., 2014; Adarsh *et al*., 2014). The suitability of chitosan as a sorbent material can be justified by following main reasons: i) low cost; ii) ease in availability; iii) high removal efficiency (Adarsh *et al*., 2014). Chitosan is mostly available in flake shape which can not be easily

separated from solution while adsorbent experiment (Abdolmaleki *et al*., 2018). To over come this problem we mixed chitosan with plant waste materials to synthesized beads.

Walnut shell being having good mechanical strength with good stability and large surface area have been used for the removal of various types of pollutents by adsoption. Walnut shell is known as a low-cost adsorbent and are widely applied in various environmental pollution control technologies, such as methanol adsorption (Yu *et al*., 2016), metal removal (Ding *et al*., 2013), dye removal (Cao *et al*., 2014) etc.

Almond shells were used for adsorption of metals (Maaloul *et al*., 2017) and antibiotics (Flores-Cano *et al*., 2016). Shells of almond have a well-developed pore structure. Cellulose content in almond shells is 38.47 %, which means that the mechanical properties might be higher than other most shells. Due to these qualities, almond and walnut being utilized together which will improve the mechanical properties of chitosan and will be helpful in forming beads for adsorption of anti-infection agents.

Chapter - 2

Review of literature

Chapter – 2

REVIEW OF LITERATURE

2.1. Antibiotic usage and production

Antibiotics have been used to treat infections and are foundational component of modern medicine. They are used for treatment of diseases caused by fungi and bacteria, and are also widely used for agricultural purpose, animal farmings and human therapy (Martinez *et al*., 2009). To encounter newer forms of microorganisms, upgraded or modified version of antibiotics are introduced in a regular fashion to tackle various ailments in plants, humans and animal community (Van Bunnik and Woolhouse, 2017). Increasing population demands increased production of antibiotics and in India during 2006-2007, it was more than 2332 Mt and production of which increases by 10% each year (Mutiyar *et al*., 2013). According to Hu, (2010), the production of antibiotics in U.S. (2003) was about 9200 tons. The European federation of animal health (FEDESA, 2001) provided the data, according to which European Union and Switzerland together consumed 13288 tons of antibiotics, out of which 65% was used as human medicine (Al- Ahmad *et al*., 2009). The China produced 210000 tons of antibiotics anually and 48% of which is applied in agriculture and livestock industry (Zhou *et al*., 2013). Global consumption of antibiotics grew more than 30% from 50 billion to 70 billion standard units in 71 different countries (Van Boeckel *et al*., 2014). Most of these antibiotics are used which are either recommended by health care suppliers or procure directly by consumers without any prescription (Kotwani and Holloway, 2011).

More than 259 antibiotics have been registered for human and veterinary use (Azhar *et al*., 2016). The usage was around 93 tons in 2002 in New Zealand which was used for veterinary purpose (Hou *et al*., 2015). There are about 41% hikes in penicillin and cephalosporin consumption in the first decade of this century (Van Boeckel *et al*., 2014). The ten yearly antibiotic consumption in livestock may increase by 120% in China, 60% in the USA and 100 % in India, to treat common infections (Van Boeckel *et al.,* 2014). The country consumes most antibiotic overall in 2010 were India with 13 billion standard units, China 10 billion standard units and US 7 billion standard

units. Looking to the overall production and consumption of the antibiotic, the fight is to curb the menace of microbial infection to human and veterinary practice. The extra ordinary effort shows a vital data regarding invention and increased production of newer version of antibiotics for the newer society.

2.2 Antibiotic disposal through different sources (hospital, veterinary, municipal and agricultural) ends up in creating pollution to aquatic ecosystem.

Largely, variety of anthropogenic activity has resulted in the leaching of antibiotics and their metabolites in aquatic ecosystem, proving one of the most hazards environmental pollution. The manufacturing units are adding antibiotics to some extent into the waste waters (Kummerer, 2009). However, emission of antibiotic from production units have found importance, as antibiotics has been detected from the effluents of these production units (Larrson *et al*., 2007). Indiscriminate use, improper handling and disposal resulted in their existence in the environment. Municipal and hospital waste, human and animal excretion consistently adding up the partially metabolized drug in the ecosystem. Over and inappropriate use of antibiotics is rampant. And negligent disposal is a major concern for the environment. Because of the chemical nature of the antibiotics they remain in the system either completely or partially metabolized. Due to this ecosystem gets polluted wherein they pose health hazard to the flora and fauna.

2.3 Antibiotics through hospital wastes

It is well accepted and known fact that pharmaceuticals are existent in hospital wastewater (Kummerer, 2009). Hospital effluents are considered as chief source of pharmecuticals in the aquatic bodies (Diwan *et al*., 2009), and are the main source in terms of pharmaceutical load generated (Al-Aukidy *et al*., 2014). The hospital waste waters are composed of effluents of different services: laboratories, radiology departments, outpatient departments, transfusion centers and wards (Verlicchi *et al*., 2012) which are the major contributors of antibiotic in the hospital effluents. The hospitals discard about 1250 million pounds of pharmaceuticals per annum as waste which are either used or unused (Brain *et al*., 2009). No regulation on the concentration limit of antibiotics into the environment has been set (Kemper *et al*.,

2008). Pharmaceuticals which are discharged in hospital wastewater are found to be higher in concentration than municipal sewages (Ashfaq *et al*., 2016). Along with health care establishments the hospital wastes are discharged directly into the urban sewer systems (Sharma *et al*., 2013). The chemical substances used by hospitals such as pharmaceuticals, disinfectants, solvents for medical purpose and many of these chemicals are being found to be resistant to waste water treatment plant (Sharma *et al*., 2013). Being resistant to wastewater treatment plants the antibiotics as parent or metabolites find their way to the environment resulting in aquatic pollution by disturbing the natural entity of water. Fluoroquinolone in hospital waste water was found in which moxifloxacin detected in higher concentration in both wastewater (224 µg/L) and sludge (219 µg/kg) (Ashfaq *et al*., 2016).

2.4 Antibiotics from animal establishments.

The consumption of veterinary antibiotics till 2010 was 63000 tons and is expected to reach 106,600 tons till 2030 (Pan *et al*., 2017). Use of veterinary antibiotics has become a matter of concern in agricultural soils (Hu *et al*., 2010) and aquaculture (Kim *et al*., 2011). Veterinary antibiotics enter into the environment either by animal excretion, by discharge of wastewater, or by way of manure application in aquaculture (Pan *et al*., 2017). About 30 to 80% of antibiotics are defecated into the environment via manure (Zhang *et al*., 2014). Veterinary pharmaceuticals are regarded as emerging contaminants (R- Navas *et al*., 2013), used worldwide, and are released incompletely metabolized in the aquatic bodies (Kolodziejska *et al*., 2013; Awad *et al*., 2014). The antibiotics used in livestock farms are used to increase the livestock productivity, suppress parasites and prevent disease caused by bacteria (Awad *et al*., 2014). Animal wastes find their way into receiving environment and contaminate water bodies as parent compound or metabolites (Sarmah *et al*., 2006). The incompletely metabolized antibiotics excreted by animals are the primary source of antibiotics in the environment (Brown *et al*., 2006), causing water to be unsafe for aquatic flora and fauna. Wei *et al*. (2011) reported the frequency of veterinary antibiotic residues from wastewaters were sulfamethazine > oxytetracycline > tetracycline > sulfadiazine > sulfamethoxazole. In another study, Hou *et al*. (2014) reported different classes of antiniotics from manure samples in Northern China. They tetracycline antibiotic was present in highest concentration in manure samples.

2.5 Antibiotics in water: the result of human activity.

The household pharmaceuticals enter municipal waste waters through three major ways, these are (1) human excreta, (2) household wastes, (3) wash-off of the human body. Generally the expired or remainder medicines are disposed down household drains. In a study, it was found that 1/3rd of total pharmaceuticals (Germany) and 1/4rd (Austria) are being disposed of through household drains (Kummerer, 2009). The incompletely digested drugs found their way to sewage treatment plant (STP) (Kummerer, 2009; Homem and Santos, 2011; Gao *et al*., 2012). The municipal waste waters become an important source of antibiotics, as 10-25% of antibiotics consumed by patients come directly from hospitals (Vlitalo *et al*., 2017), and results their widespread presence in water environment (Ekpeghere *et al*., 2017). Therefore the disposal of unused pharmaceuticals, either by toilet or by household waste is the prominent route which needs a greater attention. These unused or used medicines in the aquatic system disturb the normal functioning of the natural ecosystem.

Larsson *et al*. (2007) examined the effluents from WWTPs, near Hyderabad, India. Highest concentration of antibiotics were reported till now from these effluents. The concentration of ciprofloxacin was detected highest which was upto 31.0 mg/L.

In a study Dong *et al*. (2016) reported 19 antibiotics from conventional wastewater treatment plants from eastern China. The detection frequencies for all the target antibiotics were more than 90%. The dominant antibiotics detected were ofloxacin, clarithromycin, roxithromycin and erythromycin.

In a study conducted in France it was found that 23 antibiotics were detected from WWTP and surface waters of two head water streams. The concentration of antibiotics in WWTP was more than the domestic sources. The antibiotics fluoroquinolone were found at low concentration (Dinh *et al*., 2017).

2.6 Antibiotics gaining in water, through agricultural wastes.

Use of antimicrobial in food production has been divided into livestock production, fish farming and crop growing. (O' Neill, 2015). Farming of aquatic organisms- fish, mollusks and crustacean is in demand to supply the need of food (Rosi-Marshall, 2011). In this practice antibiotic is used for therapy and prophylaxis. Various

antibiotics used are florfenicol, premix, oxytetracycline, erythromycin, sarafloxacin, sulphonamides. Practice of antibiotics in aquaculture is a growing concern as residues of antibiotics remain in the fish products and also in the aquatic environment for an extensive period of time (O' Neill, 2015). Suggestions have been received that fishes excreted 70-80% of antibiotics consumed (Burridge *et al*., 2010). The frequent use of antibiotics for agricultural purpose results their continuous release and detection in the environment (Zhou *et al*., 2016). Manure and sludge which are also used as fertilizer for agricultural land are often contaminated with antibiotics (Kong, 2006). The wastewater treatment plants incompletely remove antibiotics (Escher *et al*., 2011), which then find their way into surface waters (Brown *et al*., 2006), resulting in aquatic pollution.

The urine samples collected from pre and primary school children were analysed for the presence of veterinary antibiotics. In 77.4 percent of the samples, urinalysis showed presence of one to four target antibiotics (Li *et al*., 2017).

Mirzaei *et al*. (2018) assessed the presence of antibiotic compounds from WWTPs in Tehran. The ciprofloxacin antibiotic detection was frequent from water samples of WWTP. Cefixime and cephalexin were the most abundant detected antibiotics in WWTP samples.

You *et al*. (2018) evaluated the presence of β-lactams and fluoroquinolones antibiotics from seven cities in China. The presence of β-lactam and fluoroquinolone antibiotics were confirmed from landfills. The highest concentration of β-lactams and fluoroquinolones were detected in Shanghai and Hangzhou respectively.

Huang *et al*. (2019) analyzed the presence of antibiotics from from land fills, tap water, WWTPs and river channels. The presence of antibiotics from ng/L to mg/L were confirmed from all the sections.

The study carried out by Gros *et al*. (2019) reported the presene of fluoroquinolone, tetracycline and pleuromutilin antibiotics from the manure amended soils.

2.7. Presence of antibiotics in water bodies

International status

Inappropriate discharge of parent antibiotic and their metabolites in the environment from the production units are of huge importance because of their detection in water

bodies. After administration to humans, animals and aquatic organisms. The metabolites are excreted to STPs, which may take their route to aquatic system. The active substances discharge with liquid manure can be washed off by rain which can also contribute towards total concentration of antibiotics in the water bodies. Hence the ultimate dead end of the parent compound and their metabolites are the aquatic system which is the important ecosystem harboring many flora and fauna**.** The majority of countries are facing water quality issues, as every source of water has been reported to be contaminated with different contaminants including antibiotics (Gothwal *et al*., 2014). The production, consumption and unsafe disposal of antibiotics are responsible for their frequent detection from every environmental matrix (Ashfaq *et al*., 2016). Several investigators have reported the antibiotic presence in different water compartments, including WWTP effluents (Chen *et al*., 2012; Jiang *et al*., 2013; Guerra *et al*., 2014), sediments (Luo *et al*., 2011; Chen *et al*., 2012; Liang *et al*., 2013; Awad *et al*., 2014), surface water (He *et al*., 2015), Ground water (Hu *et al*., 2010; Ma *et al*., 2015) and seawater (Zhang *et al*., 2013).

Landfill leachates may be responsible for the presence of pharmaceuticals for human medical care in groundwater's (Heberer, 2002). The increasing amount of proof indicates that the leaching of antibiotic compounds in ground water and deeper soil profiles. The input of antibiotics in aquatic system from agricultural fields is generally via surface runoff (Kummerer, 2009). Intensive fish farming requires antimicrobial agents to control infections, thus entry to aquatic system is direct. Such antibiotics results in high local concentration in aquatic compartment. Concentration of antibiotics was higher in hospital effluents than Municipal wastewater (Xu *et al*., 2007). Different antibiotic classes have been detected in different water systems, lincomycin detected in livestock and hospital effluents, fluoroquinolone as ciprofloxacin in hospital effluents, enrofloxin used as veterinary medicine was detected in STPs effluents, sulfonamide was found in ground water, drinking water and surface water in different concentrations (Santos *et al*., 2010). Tetracyclines and sulfonamide were detected in pig wastewater (Santos *et al*., 2010). Tylosin antibiotic has been reported in drinking water, clarithromycin, spiramycin and quinolones from waste water (Zuccato *et al*., 2010). The antibiotics present in excretory wastes are leached and will diffuse into the sediments (Cabello *et al*., 2006). There are reports of

municipal landfill leaching which contains antibiotics and end up in water bodies (Li *et al*., 2014). There are numerous other different important classes of antibiotics have been reported in water bodies they are macrolides, aminoglycosides, tetracycline's, quinolones (Brown *et al*., 2006; Martinez *et al*., 2009). Sulfonamides were found near field flood irrigation gates (Tong *et al*., 2014). Sulfadimethoxine and sulfamethazine were reported from wells, which are sources of drinking water for Washington County (Tong *et al*., 2014).

The presence of 19 antibiotics (fluoroquinolone, sulfonamide, macrolide and tetracycline) from ground water and surface water samples collected from central China were investigated. The concentration of chlorotetracycline, doxycycline and enrofloxacin were present in all water samples (Tong *et al*., 2014).

Yan *et al*. (2014) investigated the presence of antibiotics fromwaste samples of Yangtze Estuary. They selected 20 antibiotics for detection and out of which, 19 antibiotics were detected in all water samples.

The occurrence and distribution of 12 antibiotics from mainstream and tributaries of the Songhua River were evaluated (Wang *et al*., 2017). They collected surface water samples in different seasons and cefazolin and sulfamethoxazole were present in all water samples.

Zhang *et al*. (2018) investigated the presence of antibiotics from surface water samples. The frequency of diltiazem, chlorotetracycline, acetaminophen, caffeine, and ofloxacin was 100% in surface waters. Where as ofloxacin, tetracycline, carbamazepine and chlortetracycline presence was 100% in samples of upstream rivers.

The presence of macrolides, β- lactams, sulfonamides, chloramphenicol, lincomycin, monensin and furazolidon in surface waters were analyzed by Yi *et al*. (2019). The presence of antibiotics were below 82.5 ng/L in surface waters.

The occurrence, distribution and bioaccumulation of 22 antibiotics, including five macrolides, eight fluoroquinolones and nine sulfonamides were analyzed by Gao *et al*. (2012) from river water samples. The presence of roxithromycin, sulfadiazine, erythromycin and ofloxacin were reported from all water samples.

A nationwide survey was conducted by Ma *et al*. (2015) to investigate the presence of antibiotics from different wate sources. They selected 15 sampling sites and targeted most commonly used 20 antibiotics. All the 20 types of antibiotics were detected from reclaimed and ground water. The antibiotic class fluoroquinolone and sulfonamides was predominant in reclaimed water samples. Antibiotics norfloxacin and ofloxacin were detected from 90% of samples. Where as sulfamonomethoxine and trimethoprim were detected in 78% samples and oxytetracycline, doxycycline and fluoroquinolones were detected from 82% samples. Fluoroquinolones antibiotics were dominant in northern China water samples and sulfonamides from south.

The occurrence and behavior of 20 antibiotics from five classes from water samples were investigated by Chen and Zhou *et al*. (2014). The sulfonamides were detected in higher concentration in water samples out of 20 antibiotics whereas macrolides and tetracycline were found in higher concentration in sediment samples.

Burke *et al*. (2016) examined drinking water samples for the presence of 26 veterinary antibiotics which are introduced into the ground and surface water by application of liquid manure. Out of the 26 antibiotics which they analyzed 8 antibiotics i.e, trimethoprim, sulfadimidine, sulfamethoxazole, dehydrato-erythromycin, tylosin, sulfadiazine, tetracycline and sulfapyridine were identified from surface water samples. Trimethoprim were detected from shallow ground water samples.

Yao *et al*. (2017) studied the presence of 14 antibiotics from different classes and reported their presence from different water samples.

The presence of trimethoprim, oxytetracycline, sulfamethoxazole and trimethoprim antibiotics from different waste water samples were reported by Siedlewicz *et al*. (2017).

Azanu *et al*. (2018) reported antibiotic presence from lettuce and waster waters. Antibiotics ciprofloxacin, erythromycin, sulfamethoxazole, trimethoprim and cefuroxime were found in high concentration in all smples.

Lai *et al*. (2018) reported the frequency of antibiotics in the order of ibuprofen > lincomycin > flumequine > ifosfamide > cephalexin.

Looking to the above mentioned studies it can be summarized as antibiotics (parent and metabolites) paved their way to aquatic system from different anthropogenic

sources. The antibiotics are being detected in different aquatic system all over the world which remains in them for longer period of time. Their presence may affect the life of different flora and fauna which can further be selected through toxicological studies.

National studies shows that, after USA and Europe, India is the third largest pharmaceutical producer with turnover reached US$74 billion per year (Mutiyar *et al*., 2014). The organized sector of Indian pharmaceuticals consists of around 250–300 companies, with its drug exports growing 30% annually. In other words, every third pill taken in the world is manufactured by India. Among the bulk formulations, around 80% have been reported to be consumed indigenously. Production and use of large quantities of pharmaceuticals for human and veterinary applications could lead to the release of more pharmaceuticals substances into the environment (Mutiyar *et al*., 2014). On the other hand, treatment capacity of domestic sewage in India is far below the quantity of sewage generated by 1.3 billion people; only 31% of the total sewage produced (~38,254 million liters per day) in 908 cities were treated in 2008 (Mutiyar *et al*., 2014).

Despite of relatively lower levels (ng/L to μg/L) of pharmaceuticals in wastewater from WTPs that process predominantly domestic sewage, much higher concentrations (mg/L) of pharmaceutical contaminants were reported from the WTPs that process wastewater from the pharmaceuticals production facilities (Larsson *et al*., 2007). Studies conducted at the (Patancheru Enviro Tech Limited) PETL, WTP near Hyderabad, that received 1.5 MLD effluents from ~90 bulk drug manufacturers in the vicinity in Patancheru, found the highest levels of pharmaceuticals ever reported in wastewater from elsewhere in the world. Antibiotics from major classes are commonly detected at higher concentrations in wastewater from Indian WTPs that treat predominantly the domestic sewage. Antibiotics were found in drinking water and ground water bodies (Diwan 2010), and also pharmaceuticals like metronidazole ofloxacin, norfloxacin ciprofloxacin, sulphamethoxazole, tinidazole, ceftriaxone and levofloxacin were detected from hospital effluents (Diwan 2009). The presence of antibiotics (tinidazole, sulfamethoxazole, norfloxacin, ciprofloxacin, methoxytindazole, ofloxacin, and quinolones) from different water matrix were reported (Mutiyar *et al*., 2013). The presence of levofloxacin, azithromycin and

acetaminophen antibiotics from the samples of waste water treatments plants were also reported by Mohapatra *et al*. (2016). Kumar *et al*. (2019) reported the presence of carbamazepine and acetaminophen antibiotics from the waste water bodies. Sewage treatment plants from south India showed presence of trimethoprim, sulfamethoxazole, erythromycin, ampicillin and chloramphenicol antibiotics (Prabhasankar *et al*., 2016). And two tropical rivers of southwestern India have naproxen, sulfamethoxazole, chloramphenicol, ceftriaxone, and trimethoprim antibiotics in their effluents Joshua *et al*. (2020). Diwan *et al*. (2018) reported the presence of sulphamethoxazole from water and sediment samples.

2.8. Toxic effects of antibiotics

Implementation of adequate knowledge for disposal of antibiotics and waste water treatment causes the entry of these chemicals into environment especially the aquatic compartment. Toxicity studies have reported that chronic exposition is critical defining high half-life of antibiotics in water result in development of resistance and toxicity to many microorganisms. Results of various experiments showed that long term exposure of microorganisms to antibiotics cause toxic effect (kummerer *et al*., 2000; Gonzalez-Pleiter *et al*., 2013; Yang *et al*., 2013; Freitas *et al*., 2018; Wei *et al*., 2018). Toxic effect of antibiotics has clearly stated that, antibiotics are potential genotoxic agent which is proved through animal and microbial assays (Cavas and gozukara, 2005). Various tests have been designed to assess the toxicity effects of antibiotics like SOS chromo test on *E coli*, point mutation test (Ames test) on Salmonella species (Isidori *et al*., 2005). The usage of antibiotics in the human society gained its impurities for treatment of microbial infections as human medicine. However over and indiscriminate use resulted in toxicity to human system (Sekeroglu *et al*., 2012; Kayraldiz *et al*., 2015; Turani *et al*., 2015; Sekeroglu *et al*., 2016; Arslan *et al*., 2017). Higher plants also being used as the model to test the genotoxicity of antibiotics through micronucleus formation, root tip chromosomal aberration assay and sister chromatid exchange (Liu *et al*., 2012). Recent studies reported toxic effects of antibiotics on both plant species and aquatic organisms in laboratory conditions (Bauger *et al*., 2000).

International status

Flaherty and Dodson, (2005) studied the toxicity of antibiotics by using *Daphnia magna* as model organism. The antibiotic exposure had no noticeable effects on survival and reproductive parameters of *D. magna*.

Isidori *et al*. (2005) reported the toxic effects of macrolides on *D. magna*. Reproductive parameters were severly effected in the dose dependent manner.

Yamashita *et al*. (2006) reportd the reproductive toxicity of clarithromycin and levofloxacin on *D. magna* and chronic toxicity on crustaceans.

Meinertz *et al*. (2010) evaluated the chronic toxic effects of antibiotics on daphnids. More then 50% reproductive inhibition was observed in all groups and 21 day exposure to diphenhydramine hydrochloride and erythromycin showed no significant impact on survival of *D. magna.*

Ebert *et al*. (2011) reported 48 h EC_{50} value of 173 and 10.2 mg/L of enrofloxacin and ciprofloxacin on cyano bacteria, and for *L. minor* EC_{50} values were 107 and 62.5 mg/L

The toxicological effect of antibiotic oxtetracycline on Indian major carp *Labeo rohita* was evaluated by exposing them to different concentrations. Physiological variations with in the organisms were recorded after the exposure of the organism to antibiotic for a particular time period (Ambili *et al*., 2012).

Kim *et al*. (2012) reported no effects of lincomycin and acetaminophen on reproductive parameters of *D. magna* after 21 day chronic exposure.

Bartlett *et al*. (2012) reported toxic effects of sulfonamides on survival of *Hyalella azteca*. Among the sulfonamides tested, sulfasalazine was reported to be most toxic to the *H. azteca*.

Ji *et al*. (2012) evaluated the toxic effects of veterinary antibiotics using Daphnia species as model organisms. *D. magna* reproductive behavior was severly effected by antibiotics.

Martins *et al*. (2012) reported chronic toxic effect of ciprofloxacin on *D. magna* reproductive behaviours. There was no toxic effect observed on *Gambusia holbrooki* survival.

The acute and chronic toxic effects of triclosan on *Daphnia magna* was investigated by Peng *et al*. (2013). The 48 h LC_{50} of triclosan was 330 µg/l for *D. magna*. The chronic effects were observed on body length and total number of neonates of *D. magna*.

Oliveira *et al*. (2013) evaluated sub-lethal effects of amoxicillin and oxytetracycline on zebrafish. Amoxicillin resulted in early hatching and oxytetracycline had no effect on hatching of embryos.

Kołodziejska *et al*. (2013) investigated the aquatic toxicity of four veterinary antibiotics by using *Daphnia magna* as model organisms. The result demonstrated that oxytetracycline and florfenicol showed toxic effects on daphnia (EC_{50} 114 and 337 mg/L), whereas metronidazole showed no effect on neonates.

Dalla Bona *et al*. (2014) evaluated toxic effect of antibiotics on *D.curvirostris.* The EC_{50} value of efloxacin on *D.curvirostris* was 4.3 mg/L in chronic test effects on reproductive parameters were observed.

Huang *et al*. (2014) investigated the toxicity of sulfa-monomethoxine (SMM) daphnia species. The EC_{50} for *Daphnia magna* was observed to be 48 mg/L and 283 mg/L for *D. similis*. Reproductive parameters were severly effected in 21 day chronic toxicity testing.

The toxic effects of antibiotics on *Daphnia magna* in multi-generational test were investigated by Dalla Bona *et al*. (2015). The enrofloxacin showed 100 % mortality and trimethoprim 50% in F_o generation. In F_1 reproductive parameters were severly effected in 21 day chronic toxicity testing.

The study carried by Zhang *et al*. (2015) used zebrafish embryos to evaluate the acute toxicity of tetracycline antibiotic. The developmental malformations includes shorter body length, coagulated embryos, hatching delay and delayed yolk sac absorption.

Li and Lin, (2015) evaluated the acute toxicity of antibiotics on *Cyprinus carpio*. The 96 h LC_{50} value observed was 60.68 mg/L in chronic toxic effects and 40% mortality was observed after 28 days.

The study carried out by Han *et al*. (2016) reported trimethoprim antibiotic reproductive malformations and mortality on copepod *Tigriopus japonicas.*

Rodrigues *et al*. (2016) studied the acute and chronic effects of erythromycin in *Oncorhynchus mykiss*. The genotoxic effects was observed to be increasing in a dose dependent manner.

Dalla Bona *et al*. (2016) evaluated the toxic effects of enrofloxacin on growth, survival and reproduction over four generation of *Daphnia magna*. Till third generation mortality was observed to be 100%, whereas growth inhibition was observed in one group of fourth generation.

Havelkova *et al*. (2016) assessed the toxic effects of penicillin G, vancomycin and tetracycline antibiotics using *Daphnia magna.* Tetracycline showed highest toxic effect on daphnia (48h EC_{50} 8.16 mg/L), while as penicillin G was the least toxic for all test organisms.

The investigation of toxic effects of florfenicol, sulfamethazine and clarithromycin on embryo and larvae of adult zebrafish were evaluated by Ryan *et al*. (2017). No toxic effects on embryos were observed after 96 hrs of exposure. Changes in swimming activity were seen in 5-days post fertilization larvae.

The toxic effect of cefadroxil and cefradine antibiotics was investigated by Kim *et al*. (2017). They reported decrease in reproduction and growth in *Daphnia magna* and *Oryzias latipes* when exposed to cefadroxil and cefradine antibiotics

The reproductive behavior was severly effected when *C. silvestrii was treated with* florfenicol and oxytetracycline antibiotics (Freitas *et al*., 2018).

A chronic toxicity test was done to understand the potential effects of continuous exposure of *Daphnia magna* to ciprofloxacin antibiotic Nunes *et al*. (2018). No significant impacts were observed on life history parameters of *D. magna*.

Luo *et al*. (2018) performed chronic toxicity test on *D. magna* using lomeofloxacin. The effects were observed on reproduction behavior and growth of *D. magna*.

De Liguoro *et al*. (2019) observed the poisonous effects of flumequine on *D. magna*. They observed reproductive effects including birth defects, delayed hatching and mortality.

Motiei *et al*. (2019), investigated the chronic toxic effect of ciprofloxacin antibiotic on *Daphnia magn*a. Ciprofloxacin showed direct effect on growth and reproductive parameters such as first day of brood/offsprings, survival of organisms.

National scenario shows that pharmaceutical industries are located mostly in Gujarat, Maharashtra, Andhra Pradesh, Himachal Pradesh and Goa. Pharmaceutical industrial effluents have high active pharmaceutical ingredients (API) especially in India (Larsson *et al*., 2007). The effluent from a WWTP in Patancheru (Hyderabad, India) has been reported to pollute the region's waters with the highest levels of pharmaceutical residues ever detected in the aquatic environment (Lubik *et al*., 2009).

Antimicrobial-resistant bacteria and their genes have been reported from different water sources of India and their major sources are the pharmaceutical waste waters and hospital effluents that are released into the nearby water bodies without adequate treatment (Diwan *et al*., 2010). Low antibiotic concentrations in aquatic environments play an important role in maintaining resistance in bacterial populations (Gullberg *et al*., 2011). Different reports demonstrated that high concentration of antibiotics possibly triggers the development of multidrug resistant bacteria in the Indian aquatic environment, as the NDM-1 positive bacteria was recently reported in Delhi's environment (Walsh *et al*., 2011). Flaherty and Dodson, (2005) concluded that a cocktail of drugs negatively affected *Daphnia magna's* growth and reproduction and the exposure duration played a critical role in inducing toxicity. Pharmaceutical residues in aquatic environment provide lifelong exposure to aquatic organisms, and thus, their occurrences in aquatic environment are of high concern. Similar toxicity observations, i.e., harmful effects on algae, fishes and other aquatic organisms were reported by Sanderson *et al*. (2004) and Zounkova *et al*. (2010).

2.9. Differential employment of antibiotic removal strategies.

Conventional technologies include filtration, coagulation/flocculation/sedimentation, oxidation, Fenton and photon Fenton, ion exchange, activated sludge, oxidation

process, carbon filtering, constructed wetlands, nanotechnology, membrane distillation, reverse osmosis are in use for the removal of pollutants. As per Al-Aukidy *et al*. (2014) conventional WWTP are unable to remove antibiotics from sewage water. High cost functioning for the removal of antibiotics demand to look for more appropriate, convenient, cost effective removal technologies of antibiotics for a safer environmental system. Therefore, a need of efficient mechanism is in demand to prevent the aquatic organisms from harmful effect of the left over antibiotics and their metabolites. During the present investigation it was found that some of the model techniques were employed but they do possess drawbacks in their functioning some of them are highlighted as follows.

2.9.1. Membrane filtration

This technique is efficiently used for the removal of suspended contaimnents from waste waters but did not show good results in the removal of pharmecuticals. Secondarly their operational costs are also high which limits their application. Presently membrane filtration, nano filtration and reverse osmosis are used in drinking water treatment facilities, but their application in treatment of wastewater is scarce (Suarez *et al*., 2008).

2.9.2. Advanced oxidation process and ozonation

During ozonation, O_3 oxidize micropollutents directly or by the hydroxyl radicals ($HO^{\bullet}$). The purpose of advanced oxidation process is to use $HO^{\bullet}$ as a strong oxidant and ozonation disinfect the final effluent before discharge. They together are effective against different types of organic and inorganic pollutents. The investigation of its use against pharmaceuticals is scarce. Ozonation can result in by-product formation, which can have toxic properties (Ulson *et al*., 2010).

2.9.3. Electro-oxidation

This technique require high cell voltages to oxidize the pollutants in waste water plants. The operating cost is the main disadvantage and applying this technology is efficient when the effluent have sufficient conductance (Anglada *et al*., 2009).

The disadvantages of the modern technology highlights their improper functioning thereby the need of an efficient system is required for complete and safe removal of

antibiotic from aquatic environment. The choice of adsorption technology is favored due to its advantages such as facile operation, high efficiency, low cost and the easy design of adsorbents.

2.10. Adsorption technology

Adsorption is defined as the increase in concentration of a certain constituent at the surface or interface of adsorbents. Different factors which effect adsorption of adsorbent on adsorbate include pH of adsorbate, temperature, contact time, initial concentration of adsorbate and adsorbent molecules (Amouzgar and Salamatinia, 2015). Adsorption is widely acknowledged as an efficient, effective and economic method for water contamination applications for the removal of several pollutants from waste waters because of simple design, easy operation, flexibility, suitability for batch and low capital cost (Homem and Santos, 2012; Amouzgar and salamatinia, 2015; Gisi *et al*., 2016). Some low-cost adsorbents which have already used include agricultural wastes (Ahmed *et al*., 2013), Lotus stalk (Liu *et al*., 2011), walnut shell (Yu *et al*., 2016) and rice husk (Chen *et al*. 2016).

Liu *et al*. (2011) used activated carbon to remove antibiotic norfloxacin from aqueous solution. The maximum adsorption on Al_2O_3/Fe was observed at pH 5.5-6.5 on LAC. The adsorption followed Langmuir isotherm model and cation bridging, cation exchange, hydrophobic interaction and surface complexation mechanism was responsible for norfloxacin adsorption

Moussavi *et al*. (2013) studied the removal of amoxicillin by using pomegranate wood NH_4Cl-induced activated carbon. At initial concentration 50 mg/L, 0.4 g NAC/L adsorbent dosage and at pH 6, 99 % amoxicillin was adsorbed. Activated carbon (SAC) remove 55% of amoxicillin under similar experimental conditions.

Activated carbon from lignocellulose biomass *Albizia lebbeck* seed pods was used for the adsorption of fluroquinolones from waste waters (Ahmed *et al*., 2014). Different adsorption parameters were used in batch process. The removal rate of ciprofloxacin and norfloxacin was observed to be upto 96.12% and 98.13% respectively. The adsorption followed Langmuir isotherm model with maximum adsorption capacity of

131.14 and 166.99 mg/g for ciprofloxacin and norfloxacin respectively. Adsorption also followed second order kinetic model.

El-shafey *et al*. (2012) used activated carbon to remove ciprofloxacin antibiotic from waste waters. Different adsorption parameters i.e temperature, initial concentations, adsorbent dosage and pH were investigated during batch adsorption. The adsorption equillibrum was 48 hrs at pH 6.0. With the increase in temperature, wet adsorbent showed quick adsorption then dry adsorbent.

Ahmed *et al*. (2013) observed adsorption capacity of metronidazole antibiotic i.e. 180.74 mg/L on activated carbon. Followed pseudo second order kinetics and Langmuir isotherm model.

Malakootian *et al*. (2015) tested the kinetics and equilibrium studies on the biosorption of penicillin G from aqueous solution by *Azolla filiculoides*. Adsorption followed Langmuir isotherm model with adsorption capacity of 38.45 mg/g. At pH 3.0 high removal rates of penicillin G were achieved and reached 94.2%.

The activated carbon prepared from walnut shell was studied for batch adsorption of cephalexin (Nazari *et al*., 2016). The adsorption fitted better with Freundlich and Toth models. At initial pH value of 6.5, adsorption capacity observed was 233.1 mg/g. adsorption equillibrum was observed in 20 hrs. Adsorption followed pseudo second order kinetic model.

Xie *et al*. (2016) used waste paper towel activated with KOH for tetracycline removal. The adsorption capacity on activated carbon was 1661.13 mg/g. Adsorption followed pseudo-second order kinetic model.

Chen *et al*. (2016) evaluated the adsorption of tetracycline on rice husk ash (RHA). The adsorption parameters i.e, adsorption time, initial concentration, temperature and pH was assessed for tetracycline adsorption on RHA adsorbent. Adsorption equillibrum was observed within 600 minutes. Adsoption followed Langmuir isotherm model (8.37 mg/g) and second order kinetic model.

Gao *et al*. (2017) investigated the adsoptive property of modified biochar. The adsoption capacity observed was 0.0364 and 0.0867 mmol/g, respectively. Adsoption followed both pseudo first order and second kinetic models.

Kong *et al*. (2017) used magnetic biochar derived from herbal medicine waste for the adsorption of ciprofloxacin from synthetic water. Different adsorption parameters were involved during batch adsorption process. Adsorption followed Langmuir isotherm model with adsorption capacity of 68.9 mg/g, and pseudo-second-order kinetic model.

Zeng *et al*. (2017) used rice husk biochars for ciprofloxacin, doxycycline hydrochloride and tetracycline hydrochloride removal from synthetic water. Adsorption followed second order kinetics and Langmir isotherm model.

Another study was conducted for the adsorption of ciprofloxacin and doxycycline from water by using modified rice straw (Zeng *et al*., 2018). The parameters of antibiotic adsorption i.e, humic acid, contact time, initial antibiotics concentration, biochar dosage, pH and background electrolytes were studied. Biochars produced at high-temperature (700 ^{o}C), have higher adsorption capacity then biochar produced at low-temperature (300–500 ^{o}C).

Okoli *et al*. (2018) used starch nanocomposite as adsorbant material for the adsorption of tetracycline. Followed pseudo second order kinetic model.

Jang *et al*. (2018) investigated the adsorption tetracycline on activated biochar. While comparing with previous results, adsorption on activated BC was observed to be higher.

Fan *et al*. (2018) evaluated the adsopriton of tetracycline on biochar prepared from rice straw. The adsorption followed the order: RSBC700 > RSBC500 > RSBC300. pH had no effect on adsorption. The adsorption follows Langmuir isotherm model (50.72 mg/g) and second-order kinetic model.

Li *et al*. (2018) used peanut shells to remove antibiotics from aqueous solution during batch adsorption experiment. The adsorption followed Henry linear adsorption model and pseudo-second order kinetic model.

The carbonaceous material derived form saw dust was used for the adsorption studies of antibiotics. Adsorption of tetracycline and sulfamethoxazole follow Langmuir isotherm model with adsorption capacity of 270.53 mg/g, 295.06 mg/g respectively.

Adorption also followed pseudo-second order kinetic model and adsorption was endothermic (Ahsan *et al*., 2018).

Ali *et al*. (2018) used NaY zeolite, which was synthesized from wheat straw ash and was used as adsorbent material for the removal of antibiotics. Adsorption followed Langmuir isotherm model with adsorption capacity of 201.77, 218.51, and 230.69 mg/g respectively. Adsorption followed pseudo-second order kinetic model.

Feng *et al*. (2018) prepared biochar from luffa sponge and used it for adsorption of norfloxacin from aqueous solution. Almost 99.86% of antibiotics were removed with adsorption capacity of 250 mg/g.

Duan *et al*. (2019) chemically modified *Calotropis gigantea* fiber (CGF) by using $NaClO_2$ to improve its hydrophilicity which was used to remove antibiotics were aqueous solution. The adsorption capacity observed was 70 mg/g at pH 6.0.

Movasaghi *et al*. (2018) used oat hull for ciprofloxacin adsorption. The adsorption capacity obtained was 83 mg/g at the pH 7.0 and at temperature 318 K. The Freundlich model was the best to simulate the experimental data at all the three temperatures among the tested models.

Bansal, O. P. 2012 Thermodynamics of equilibrium adsorption of antibiotics by clay minerals and humic acid–clay complexes. *National Academy Science Letters* ***35*** (2), 109-114.

Adsorption Experiments were also carried by Indian researchers for the removal of antibiotics by using different materials. Pachauri *et al*. (2009) used powdered activated carbon (PAC) in a batch mode and granular activated carbon (GAC) in a continuous packed bed column for the removal of amoxicillin antibiotic. Batch adsorption using PAC showed 70% removal on PAC and 80% on GAC column. Bansal, (2011) observed adsorption in the order tetracycline > chlortetracycline > oxytetracycline on montmorillonite, humic acid and humic acid-montmorillonite complex. In an another study Maheshwari *et al*. (2013) used activated alumina (AA) and coal fly ash (CFA) for the Ciprofloxacin hydrochloride (CPH) from its aqueous solution. The optimal pH for CPH adsorption was found to be 4. Adsorption of CPH was well described by Freundlich isotherm. Manjunath *et al*. (2019) used potassium hydroxide (KOH) activated *Prosopis juliflora* activated carbon (KPAC) for the

removal of sulfadiazine (SDZ), metronidazole (MET) and tetracycline (TET) antibiotics. The results showed that maximum sorption capacity of KPAC was 18.48, 25.06 and 28.81 mg/g for removal of SDZ, MET and TET, respectively, in single-component adsorption system.

Chitosan attracted the attention to researchers because of its cost effective and environmental friendly technology for bioremediation of soil and water pollution. The unique features of chitosan as an adsorbent comes from its structural and chemical properties (Mahaninia *et al*., 2016). Chitosan was used for removal of wide ranging waterborne contaminants including organic and inorganic pollutants viz. Dyes (Huang *et al*., 2013; Bulut *et al*., 2014; Dotto *et al*., 2015; Li *et al*., 2015; Peng *et al*., 2015; Kyzas *et al*., 2015; Mubarak *et al*., 2016; Habiba *et al*., 2016; Albadarin *et al*., 2017; Naseeruteen *et al*., 2017; Li *et al*., 2017; Banerjee *et al*., 2017; Marrakchi *et al*., 2017; Subramani *et al*., 2017; Vaz *et al*., 2017; Ma *et al*., 2018; Sun *et al*., 2018; Vieira *et al*., 2018; Jawad *et al*., 2019; Kim *et al*., 2019), Metals (Igberase *et al*., 2014; Adarsh *et al*., 2014; Cadaval *et al*., 2015; Liao *et al*., 2016; Mende *et al*., 2016; Frantz *et al*., 2017; Kong *et al*., 2018; Mishima *et al*., 2018; Saleh *et al*., 2019; Sethy *et al*., 2019; Sessarego *et al*., 2019), pharmaceuticals (Dai *et al*., 2012; Kyzas *et al*., 2014; Chang *et al*., 2015; Liang, *et al*., 2019; Tzereme *et al*., 2019), Antibiotics (Adriano *et al*., 2005; Caroni *et al*., 2009; Priya, *et al*., 2016; Huang *et al*., 2017; Lu *et al*., 2017; Gupta, *et al*., 2017; Afzal *et al*., 2018; Bai *et al*., 2018; Danalıoglu *et al*., 2018; Wang *et al*., 2018; Abdolmaleki, *et al*., 2018; Luo *et al*., 2019; Ma *et al*., 2019; Privar *et al*., 2019; Soares *et al*., 2019; Wang *et al*., 2019). It is chemically more versatile than cellulose and chitin due to hydrophobicity, biodegradability, biocompatibility, anti-bacterial properties and because of functional groups. Cross-linking is a practice to give chitosan mechanical strength to be used in water treatment applications. Various cross- linking agents like ethylene glycol diglycidyl ether, glutaraldehyde and epichlorohydrin are generally used to make chitosan chemically and mechanically stable. (Sowmya *et al*., 2014).

Walnut shell is known as a low-cost adsorbent (Kamar and Nechifor, 2015). They are widely applied in various environmental pollution control technologies (Yu *et al*., 2016), methanol adsorption (Yu *et al*., 2016), metal removal (Ding *et al*., 2013; Pashaei *et al*., 2017; Halysh *et al*., 2018; Lu *et al*., 2019; Zbair *et al*., 2019; Liu *et al*.,

2020), dye removal (Aydin *et al*., 2009; Celekli *et al*., 2012; Cao *et al*., 2014; Hashemian and Shayegan, 2014; Dahri *et al*., 2014; Ojo *et al*., 2018; Chunli *et al*., 2018; Liu *et al*., 2018; Miyah *et al*., 2018), antibiotic removal (Nazari, *et al*., 2016; Teixeira *et al*., 2019;), pharmaceuticals (Lember *et al*., 2017). The Indian state of Jammu and Kashmir alone accounts for > 98 percent of India's total production of walnuts (Sharma *et al*., 2014).

Almonds belongs to the Rosaceae family and is one of India's most important temperate nut crops, mainly grown in the Kashmir Valley (Kumar *et al*., 2015). Almond shells are inexpensive, abundant and readily available lignocellulosic material (Kumar *et al*., 2015). Almond shells can be used as sorbent: because the studies have shown that they were used for adsorption of metals (Bulut and Tez, 2007; Demirbas *et al*., 2008; Ronda *et al*., 2013; Nasseh *et al*., 2016; Cataldo *et al*., 2017; Nasseh *et al*., 2017; Maaloul *et al*., 2017; Yuksel and Orhan, 2018; Maaloul *et al*., 2020), dyes (Thitame and Shukla, 2015; Bouaziz *et al*., 2015; Hazzaa *et al*., 2015; Maaloul *et al*., 2015; Saeed *et al*., 2015; Thitame and shukla, 2016; Saber-Samandari, *et al*., 2016; Jabli *et al*., 2017; Bordbar, 2017; Yildiz, 2017; Ahsaine *et al*., 2018; Liou and Lin, 2019; Zbair *et al*., 2018; Yuksel and Orhan, 2019) . The almond shell cell walls are made up of cellulose, silica, lignin and carbohydrates with hydroxyl groups in their structures. (Hashemian and Shayegan, 2014) antibiotics (Nazari *et al*., 2016).

Chapter - 3

Hypothesis

Chapter – 3

HYPOTHESIS

Adsorption technology proved an effective way to remove pollutants, due to their ease of operation and comparable low cost of application. In recent years low cost adsorbents including plant and agricultural wastes has been used by a number of researchers for the removal of pollutants. Chitosan considered as low cost adsorbent are extensively studied as sorbents for the removal of wide range of waterborne contaminants including organic and inorganic pollutants viz. dyes, antibiotics and metals. Walnut shell an abundant agricultural residue with good chemical stability, large specific surface area, high mechanical strength and easy regeneration has been successfully used in the removal of pollutants by adsorption. Walnut shell is known as a low-cost adsorbent. They are widely applied in various environmental pollution control technologies, methanol adsorption, metal removal, dye removal. Almond shells have a well-developed pore structure and were also reported to be used for adsorption of metals, antibiotics etc. Chitosan is mostly available in flakes or powder form which cannot be easily separated from water after adsorbent experiment and secondly, it also causes further deterioration of the water quality. Thus, use of chitosan is being enhanced by framing it into beads or sheet forms which can easily be removed from water matrix and can be reused for adsorption. But again the drawback with chitosan bead is its poor mechanical strength. Therefore, the focus is to mix walnut and almond shell powder with chitosan for hybrid bead formation as the higher cellulose and lignin content in almond shells and walnut shells will provide better mechanical properties. Due to these qualities, almond and walnut shell being utilized together with chitosan which will improve the mechanical properties of adsorbents. So it is our understanding that through the study, combination beads made from chitosan, walnut shell and almond shell will improve the mechanical strength and also the removal capacity of beads. The synthesized beads will be reused after adsorption experiments.

Chapter - 4

Objectives

Chapter – 4

OBJECTIVES

The brief objectives of our research are:-

- To evaluate the toxicity of antibiotics by using *Daphnia magna* and *Danio rerio*.
- Preparation and characterization of different combination beads (chitosan + walnut shell and/or almond shell).
- Adsorption kinetics of antibiotics from water by using prepared combination beads.

Chapter - 5

Material and methods

Chapter – 5

MATERIAL AND METHODS

Antibiotic contamination in water bodies could be an issue that is anticipated to gain more consideration in the nearest future, as antibiotic usage is increasing worldwide and annual amount of antibiotic usage has reached 100,000 to 200,000 tons. In 76 countries, the consumption of antibiotics from 2000-2015, increased 65% and the value reached 42 billion defined daily doses in 2015. There are predictions that global antibiotic usage will increase 200% till 2030 than in 2015. The antibiotics are not completely metabolized in our body and in the bodies of animals, due to which a high percentage of antibiotics are excreted into water bodies through urine and faeces, which has significant impacts on biotic and abiotic components in aquatic environment. Over the past few decades, *Daphnia magna* and *Danio rerio* have increasingly used as toxicity testing model organisms. In order to evaluate the ecological risk of cephalosporin (cefixime, cefotaxime) and fluoroquinolone (gemifloxacin, gatifloxacin) antibiotics. The effects on reproduction, survival and growth on *D. magna* were assessed over a period of 21 days. The *Danio rerio* eggs were also used to evaluate the developmental toxicity of cephalosporin and fluoroquinolone antibiotics. Among the available antibiotic removal methods, adsorption has gained much attention due to its effectiveness, feasibility and ease in operation. The research work was carried out to evaluate the acute and chronic toxicity of *D. magna* and mortality and malformations in *Danio rerio* embryos when exposed to different antibiotic concentrations. The antibiotics were also removed from synthetic waters by using beads prepared from plant waste materials.

The methodology have been divided into three parts. Part I deals with testing procedure for *Daphnia magna*. Part II discussed the testing procedure for *Danio rerio*. Part III deals with preparation, characterization of beads and batch adsorption procedure of antibiotics by using these prepared beads.

PART I

5.1. Materials

5.1.1. Test chemicals

The antibiotics cephalosporin (cefixime, cefotaxime) and fluoroquinolone (gemifloxacin, gatifloxacin) were purchased from Yarrow Chemicals (India) with purity higher than 98%. Acetic acid, sodium hydroxide and glutaraldehyde were purchased from LOBA chemicals (India). The reagents were used as received.

5.2. *Daphnia magna* toxicity testing

5.2.1. Daphnia as model organism

Daphnia magna have numerous characters which make them suitable for toxicity testing and ecotoxicological research (Kim *et al*., 2017). They have shot life sycle, reproduce by parthenogenesis, high fecundity, small size and ubiquitous occurrence (Lv *et al*., 2018). They are known as good toxic model organisms due to their high sensitivity to aquatic pollutents and they are energy link between primary producers and secondary consumers (Silva *et al*., 2019).

5.2.2. Maintenance of test organism

The master culture of *Daphnia magna* was regularly sub-cultured in the laboratory. The daphnids were cultured and maintained in reconstituted water medium. *Daphnia magna* were maintained at temperature 20±1°C with photoperiod of 16L: 8D cycle. The cledoceran culture was fed daily with yeast and algae, renewed water twice a week and similar size of *Daphnia magna* (< 24 hours) were used for this study (Lv *et al*., 2018).

5.2.3. Test chemicals solution preparation

The standard stock solutions of antibiotics (500 mg/L) were prepared from which test solutions were prepared for each antibiotic by successive dilution. The solutions were stored in a dry and dark place, at above 18ºC for no longer than 2 days before use.

5.2.4. Range finding test

The ranage finding test was performed to narrow the exposure concentrations of antibiotics. For that neonates were treated with 1, 10, 100, 1000 and 2000 mg/L of

antibiotic concentration along with control. The exposure conditions were kept same as applied for definite tests. But the daphnids were used lesser in number and duration of test was also reduced.

5.2.5. Acute toxicity test

The modified US, EPA 2002 standard procedure was used for the acute toxicity testing. Based on range finding test, the test concentration used in the acute test was as: 0, 2.5, 8, 25, 80 and 250 mg/L for cefixime and cefotaxime antibiotics and 12.34, 37.03, 111.11, 333.3 and 1000 mg/L for gemifloxacin and gatifloxacin antibiotics. A total of 5, *D. magna* neonates (24 hrs) were treated per concentration. Throughout the tests daphnids were not fed and test solution was also not renewed. The immobility of *D. magna* was recorded at 48 hrs and neonates which shows no response within 15s gentle agitation were considered as immobile. After 48 hours of exposure, the concentration of antibiotics that cause 50% immobility (EC_{50}) was calculated. The tests were performed in triplicate.

5.2.6. Chronic exposure of *D. magna*

For the chronic toxicity testing, the *D. magna* were exposed to cefixime (0, .70, 2.1, 6.3, 18.19 and 56.7 mg/L), cefotaxime (0, 0.20, 0.60, 1.8, 5.4 and 16.2 mg/L), gemifloxacin (0, 3.5, 10.5, 31.5, 94.5, 283.5 mg/L) and gatifloxacin (0, 2.5, 7.5, 22.5, 67.5, 202.5 mg/L) antibiotics. The toxicity test was carried out for 21 days with 10 individual replicates.The water quality parameters were measured on regular basis and the medium were changed daily. The endpoints analysed along the test were the survival (%), number of young per female/brood and first day of brood.

5.2.7. Statistical analysis

The experiments were repeated thrice independently. The value of 48h EC_{50} (immobility) was calculated using the probit method (US EPA Probit Analysis Program, Ver. Oh, 1.5). One way analysis of variance with Tukey's multiple comparisons was carried out using the SPSS software (Ver. 15.0).

Part II

5.3. *Danio rerio* toxicity testing

5.3.1. *Danio rerio* as toxicological model

Zebrafish is currently considered an excellent model organism in various biomedical fields (Nishimura *et al*., 2016). Zebra fish have some important characteristics which makes them most popular toxicity testing model organisms. They have high reproduction capacity, transparent embryos, small size and quick development (Chakraborty *et al*., 2016).

5.3.2. Zebra fish (*D. rerio*) maintenance (Zhu *et al*., 2008).

OECD, 2006 guidelines were followed for the toxicity testing. *D. rerio* adults were obtained from Whizbang Bioresearch Chennai. They were kept in aquarium under following laboratory conditions i.e. temperature 26±1◦C, light/dark cycle 14-h/10-h and male/female sex ratio 2:1. Fishes were regularly fed with dry flake food. The turning on light trigger the spawning in fishes and completes within 30 min. The eggs collected were from glass aquarium and they were cleaned to remove the residue on the egg surface. The fertilized eggs were picked out under a stereomicroscope for the use of experiment.

5.3.3. Test media

The reconstituted water was used as the medium for all the solutions during the experiments. The standard stock solutions of antibiotics (10 mg/mL) were prepared by dissolving required amount of respective antibiotic, from which test solutions were prepared for each antibiotic by successive dilution. The solution was stored in a dry and dark place, at above 18ºC for no longer than 2 days before use.

5.3.4. Exposure process

The acute toxicity test started within 1.5 hpf and intact egges were selected for testing. The multi well plates were filled with 2 ml distilled water and each well also contain one zebra fish embryo (**Fig. 1**). Actually, twenty wells were added with antibiotic concentration for each well and the remaining four wells were prepared similarly with 2 ml distilled water. The transparent plastic film was used to cover the 24-well plates and the experiment embryos were placed in an illumination incubator (Zhang *et al*., 2018).

5.3.5. Evaluation of developmental effects

The developmental toxicity was observed at 24, 48, 72 and 96 hours post fertilization (hpf). Morphological characters and embryotoxicity were evaluated using an inverted microscope (Selderslaghs *et al*., 2009). The various end points monitored include tail extension, coagulation, lack of heat-beat, pigmentation, eye development (Webinar *et al*., 2014).

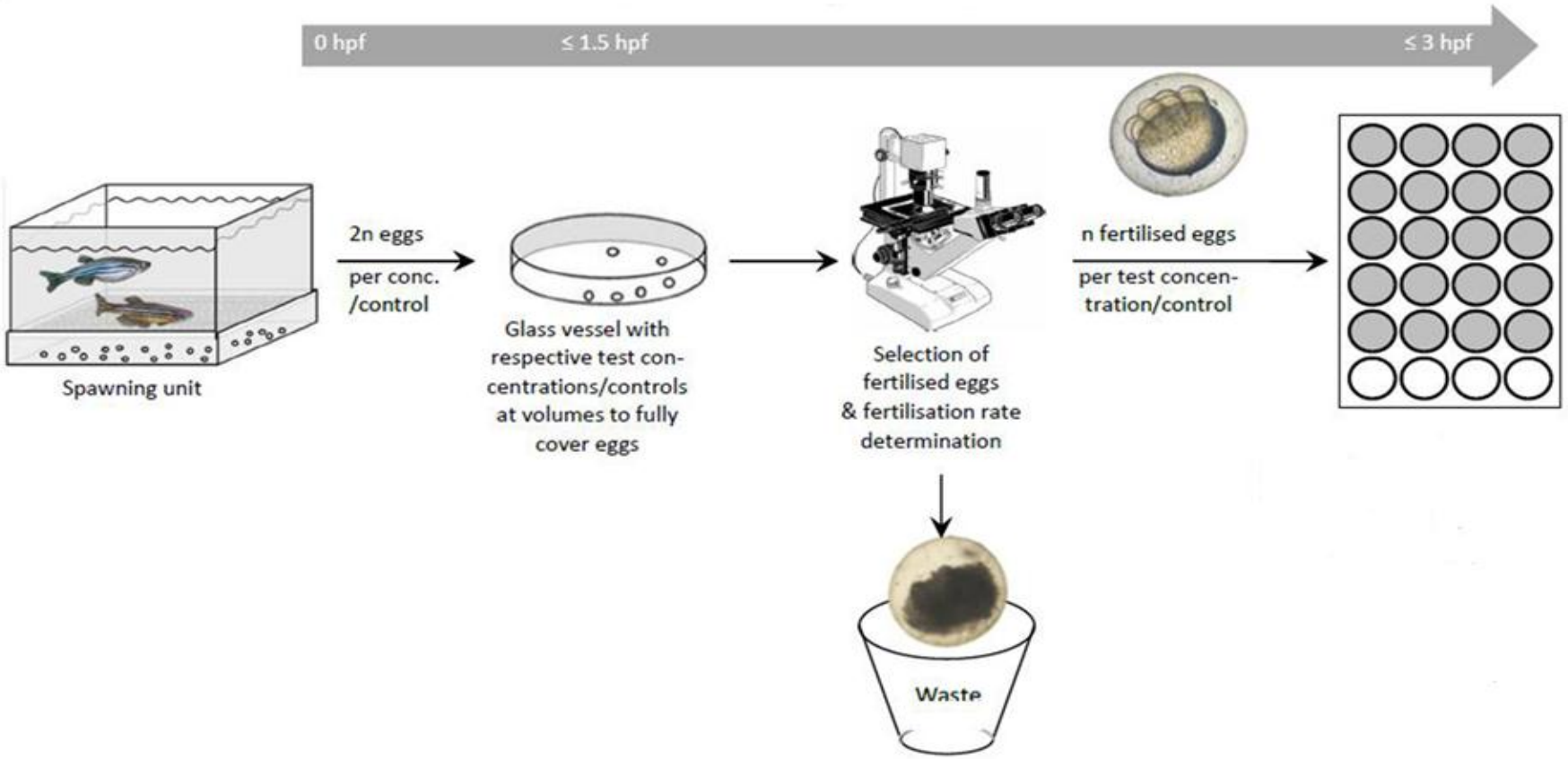


Fig. 1: Experimental design of zebra fish developmental assay.

5.3.6. Statistical analysis

The experiments have been repeated three times independently and the average values and standard deviation have been used.

Part III

5.4. Adsorption experimentation

5.4.1. Materials

5.4.2. Chemicals

The antibiotics cephalosporin (cefixime, cefotaxime) and fluoroquinolone (gemifloxacin, gatifloxacin) were purchased from Yarrow Chemicals (India) with purity higher than 98%. Acetic acid, sodium hydroxide and glutaraldehyde were purchased from LOBA chemicals (India). The reagents were used as received. Chitosan was purchased from Himedia. Walnut and Almond shells were purchased from local market.

5.4.3. Preparation of antibiotic solution in aqueous medium

The antibiotics used for adsorption studies were commercially available as fine powder. The standard stock solutions of antibiotics were prepared (500 mg/L) by dissolving required amount of respective antibiotic, from which test solutions were prepared for each antibiotic by successive dilution. The solution was stored in a dry and dark place at 18ºC for no longer than 2 days before use.

5.4.4. Preparation of Adsorbent

Chitosan was used without further purification. Walnut and almond shells were collected from local market and washed twice with distilled water to remove the dust, dried at 105 ºC for 24 hrs, then grinded, sieved separately and packed in zipper packs for further use. Chitosan, walnut and almond shell powder (in combination- AWC 2:1:1, CAW 2:1:1, WAC 2:1:1) were dissolved in glacial acetic acid (2.0%). The solutions were agitated by magnetic stirrer (8–10 h) at room temperature (23 ± 2 ^{o}C). Then with the help of syringes the solution was released drop wise into NaOH (0.5M) to form spherical beads. The beads were kept for minimum 16 h in NaOH for imbibition. For activation purpose the beads (1.5 g) at pH 5 were treated with 15 ml

glutaraldehyde solution (2.5%). The beads were stirred (150 rpm) for 3 h at room temperature. It was followed with washing of the activated beads to remove unreacted glutaraldehyde until a neutral pH is obtained and finally dried the beads in hot air oven at 50 ºC for 24 hrs and stored in air tight bottles for further use (**Fig. 2**).

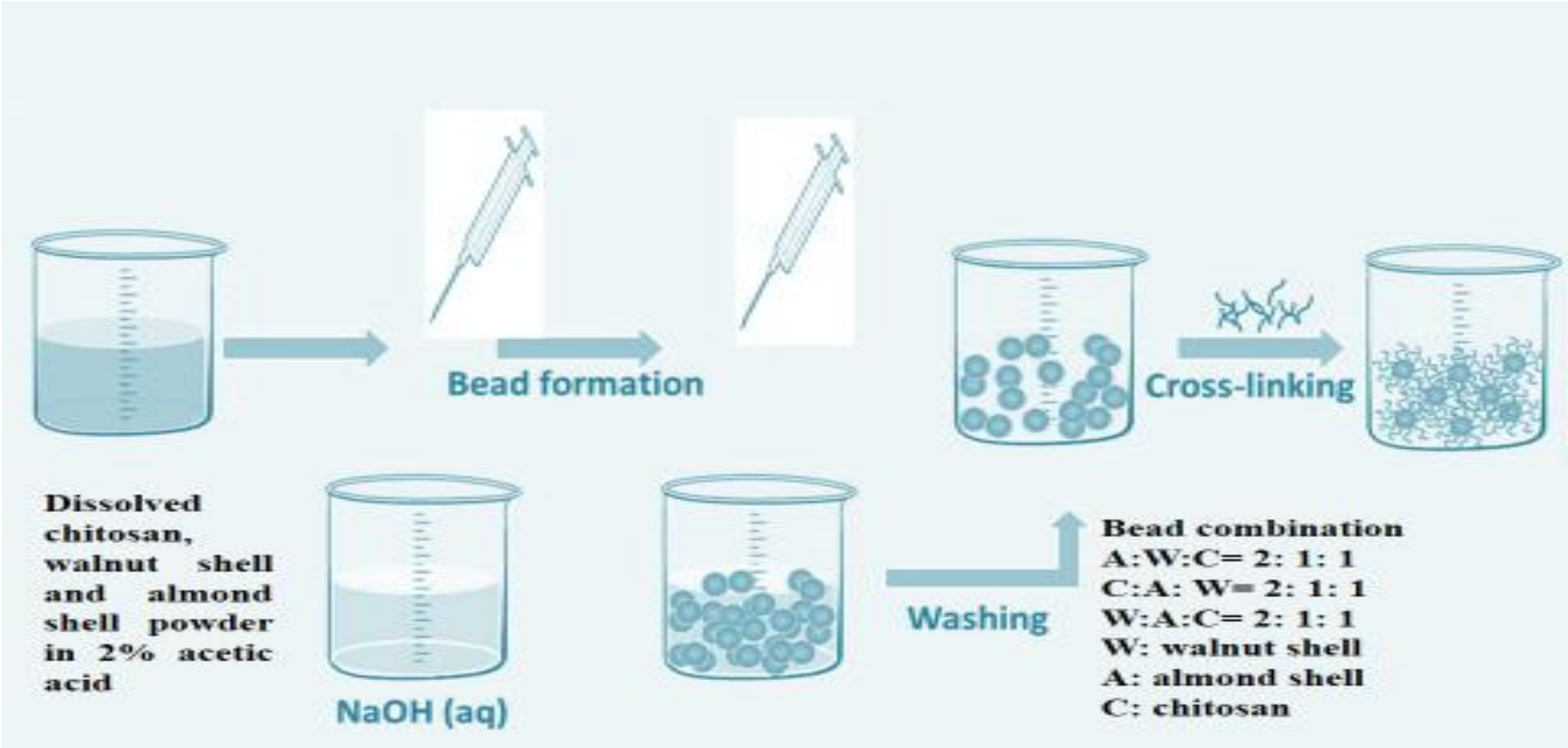


Fig. 2: **Prepration of beads from plant waste material**

5.5. Characterization of prepared beads

5.5.1. Scanning electron microscopy (SEM)

The morphology of beads were studied by scanning electron microscope (SEM-JEOL 6100) and the images of samples are produced by focused beam of electrons on adsorbent material. SEM analysis was used to study the surface texture of prepared adsorbent beads and to determine morphology of adsorbents surface.

5.5.2. Fourier Transform Infrared Spectroscopy (FTIR)

The beads were characterized using FT-IR spectrophotometer (Shimadzu-8400) in range 4000-400^{-1} cm. The surface characterization of adsorbents were done to determine the functional groups which plays an important role in adsorption.

5.5.3. Energy Dispersive X-Ray Spectroscopy (EDX)

Energy dispersive X-ray spectroscopy was used to determine the elemental composition of adsorbent beads. This technique is used in combination with scanning electron microscopy (SEM). EDX is a technique utilizes X-rays that are released from the sample when bombarded by the electron beam.

5.6. Methods

5.6.1. Batch experiments

The adsorption efficiency of the prepared chitosan, almond shell and walnut shell beads were investigated by batch adsorption studies. In a conical flask (250 ML), 0.1g beads were agitated along with antibiotics (10-50 mg/50ml) at 150 rpm 25±1^{o}C temperature. After attaining the equilibrium the adsorbent was separated and aqueous phase containing antibiotic were examined by employing UV- spectrophotometer. The parameters included different contact time (30-180 min), pH (3-11), adsorbent dosage (0.1- 1.0 g) and initial concentration (10- 50 mg/L) were examined for their effect on antibiotic adsorption (Duan *et al*., 2019).

The removal efficiency of antibiotics by prepared beads is calculated according to the equations as follows (Kumar *et al*., 2017):

$$\% \text{ removal} = \frac{C0-Ce}{Ce} \times 100$$

Where Co (mg/L) and Ce (mg/L) were the initial and equilibrium concentration of the target contaminant respectively. All experiments were conducted in triplicates.

5.6.2. Effect of variable parameters

To select the most appropriate quantity of adsorbent parameters for effective adsorption of antibiotics from solution, experiments were conducted to determine the effect of the different adsorbent parameters on antibiotic removal.

5.6.2.1. Dosage of adsorbents

Different doses consisting of 0.1 to 1.0 g/50ml of the adsorbent mixed with the fixed concentration of antibiotics and the mixture was agitated in a mechanical shaker while holding all other variables constant. The percentage of adsorption for different doses was determined.

5.6.2.2. Initial concentration

Experiments were performed with different initial antibiotic concentrations ranging from 10 to 50 mg/L in order to determine the rate of adsorption. All other factors were kept constant.

5.6.2.3. Contact time

The effect of contact time (30-180 min) between the adsorbent and adsorbate on the removal of the antibiotics was determined by maintaining initial concentration, dosage and pH constant.

5.6.2.4. Initial pH

Adsorption experiments at a wide range solution pH (3-11) were performed. The pH of medium was maintained by adding required amounts of sodium hydroxide and hydrochloric acid solution. The other parameters were kept constant while carrying out the experiments.

3.7. Adsorption isotherms

Adsorption isotherms are used to show the relationship between the amounts of adsorbate adsorbed by the adsorbent (Qe) and the adsorbate concentration remaining in the solution after the system has reached to the equilibrium state (Ce) at a constant

temperature (Kumar *et al*., 2018). Numbers of adsorption isotherm equations were proposed by different investigators.

The frequently used isotherms are:

- Langmuir adsorption isotherm

According to Langmuir isotherm, the adsorption occurs on a homogenous surface by single-layer sorption without any interaction between adsorbed ions. Only a fixed number of adsorption sites are available on the adsorbent surface, all of which have the same energy and adsorption is reversible.

The linear equation of this model is given as follows:

$$\frac{1}{\mathrm{Qe}} = \frac{1}{Q} + \frac{1}{bQCe}$$

where Q denotes maximum adsorption at monolayer (mg g^{-1}), Ce as equilibrium antibiotic concentration (mg L^{-1}), qe as the amount of antibiotic adsorbed per unit weight of the beads at equilibrium concentration (mg g^{-1}) and b is the Langmuir constant (L mg^{-1}) for affinity to the binding sites which is also a measure of the energy of adsorption (Kumar *et al*., 2017).

- Freundlich adsorption isotherm

Freundlich model states the uptake of adsorbate occurring on a heterogeneous surface which is represented by the linear as below;

$$\log \mathrm{Qe} = \log \mathrm{K_f} + \frac{1}{n} \log \mathrm{Ce}$$

where K_f is the Freundlich constant, n is the heterogeneity factor of the volume and intensity of the adsorption. K_f and n can be determined from a linear plot of log qe against log Ce (Kumar *et al*., 2017).

3.8. Adsorption kinetics

The study of adsorption kinetics provide valuable information regarding the reaction pathways of antibiotics. It also determines the rate of antibiotic update. Adsorption of antibiotics depends on both physical and chemical characters of adsorbents, which is normally controlled by three consecutive diffusive transport processes: (1) diffusion from bulk solution to the film surrounding the adsorbent particles, i.e., external

diffusion or film diffusion, (2) diffusion from the film to the adsorbent surface and (3) from the adsorbent surface to the internal sites followed by binding of the adsorbate ions onto the active sites.

In order to analyze the rate of adsorption of antibiotics on prepared beads, the Lagergren first order and pseudo–second–order were applied to adsorption data.

The Lagergren pseudo–first–order kinetic rate equation for the adsorption of liquid–solid system is based on solid uptake capacity. According to this model the overall adsorption rate is directly proportional to the difference in the saturation concentration and the amount (cumulative) of solute uptake with time.

The linear equation of Lagergren pseudo-first order is as below:

$$\text{Log (Qe - Qt)} = \log Qe - \frac{K1}{2.303}t$$

While, linear form of Lagergren pseudo-second order equation is:

$$\frac{t}{Qt} = \frac{1}{h} + \frac{t}{Q2e}$$

Where Qe and Qt are the antibiotic amount adsorbed (mg/g) by adsorbent at equilibrium and at time t (min). k^1 ($min.^{-1}$) represents rate constant for Lagergren pseudo first order, $h = k_2Q^2_{2e}$ and k_2 (in mg/g/min) denotes the rate constant for Lagergren pseudo second order (Kumar *et al*., 2017).

3.9. Data Analysis

The experimental data were analyzed using origin (version 8) Computer Software. The goodness of fit was discussed using correlation coefficient r and standard deviation (SD).

Chapter - 6

Results and discussion

Chapter – 6

RESULTS AND DISCUSSION

6.1. *Daphnia magna* toxicity testing

Toxicity tests are used to assess whether a chemical present in the aquatic environment is harmful to aquatic plants and animals. Typically acute and chronic tests are carried out for toxicity testing. Acute tests are comparatively shot in duration, simple, with low cost and are regularly used to conduct fast toxicity assessments or to evaluate the relative sensitivity of different organisms (Lomba *et al*., 2020). The chronic tests are more complicated then acute tests and take more time. Additionally, chronic toxicity tests are designed to collect data on teratogenicity (ability of a substance to cause body malformations) or carcinogenesis. In this section, we will discuss the findings obtained for the toxicity of studied antibiotics in biological model *D. magna*.

6.2. Acute toxicity of cephalosporin (cefixime and cefotaxime) and fluoroquinolone (gemifloxacin and gatifloxacin) antibiotics to *D. magna*

In aquatic ecotoxicology, cladocerans are considered as a key toxicity model. These are abundant in freshwater bodies and are feeding on phytoplankton (Tian *et al*., 2019). In addition, because of their sensitivity to water pollution, pollutents are expected to alter the behavior of these organisms making them more susceptible to the aquatic environment in which they live (Lomba *et al*., 2020).

To investigate the aquatic toxicity of antibiotics, the acute toxicity test was performed on *D. magna* neonates in a static exposure for 48h, using modified US, EPA 2002 standard procedures. The neonates were divided into control and treatment concentrations groups. The daphnids were not fed during exposure period and testing were done in triplicates. In this study, the acute toxicity of cephalosporin (cefixime and cefotaxime) antibiotics using *D. magna* were evaluated at different antibiotic concentrations (**Table 1**). A clear dose- response relationship could be observed (percentage of immobility organisms). The 48h-EC_{50} of cefixime and cefotaxime against *D. magna* was 77.92 mg/L and 25.82 mg/L respectively. In case of fluoroquinolone antibiotics the EC_{50} value showed that *D. magna* was more sensitive

to gatifloxacin than gemifloxacin antibiotic in short period of exposure. The EC_{50} value for gatifloxacin was 330.8 mg/L after 48 hrs and for gemifloxacin was 489.42 mg/L respectively (**Table 2**).

Among the cephalosporin and fluoroquinolone antibiotics, *D. magna* was more susceptible to cefotaxime (25.82 mg/L) antibiotic. The pollution level of antibiotics in water bodies are usually detected in ng/L (Li *et al*., 2020), which is much lower than the observed 48h-EC_{50} value in our studies. However, due to frequent detection of this antibiotic class in water bodies, long term effect could cause sub lethal effects in the aquatic organisms. The sub lethal effects of our studied antibiotics on *Daphnia magna* are not that much studied under different exposure pathways.

Table 1: Acute toxicity test results of cefixime and cefotaxime antibiotics on *D. magna*.

Cefixime						
Concentration (mg/L) ⟶ Exposure time (48 hrs) ↓	**Control**	**2.5**	**8**	**25**	**80**	**250**
A (a/d)	4/1	5/0	5/0	2/3	3/2	1/4
B (a/d)	5/0	3/2	4/1	5/0	1/4	0/5
C (a/d)	5/0	4/1	4/1	3/2	2/3	2/3
D (a/d)	5/0	5/0	4/1	5/0	3/2	3/2
% mortality	5	15	15	25	55	70
Cefotaxime						
A (a/d)	4/1	5/0	4/1	2/3	1/4	0/5
B (a/d)	5/0	3/2	4/1	2/3	1/4	2/3
C (a/d)	5/0	5/0	4/1	3/2	2/3	0/5
D (a/d)	5/0	4/1	4/1	3/2	2/3	0/5
% mortality	5	15	20	50	70	90

(A, B, C, D represents different experimental groups; a/d denotes number of alive (a) and dead (d) animals in each group)

Table 2: Acute toxicity test results of gemifloxacin and gatifloxacin antibiotics on *Daphnia magna*.

Gemifloxacin						
Concentration (mg/L) → Exposure time (48 hrs) ↓	**Control**	**12.34**	**37.03**	**111.1**	**333.3**	**1000**
A (a/d)	5/0	5/0	5/0	3/2	4/1	1/4
B (a/d)	4/1	4/1	2/3	4/1	¼	1/4
C (a/d)	5/0	4/1	4/1	5/0	2/3	4/1
D (a/d)	5/0	5/0	4/1	2/3	3/2	3/2
% mortality	5	10	25	30	50	55
Gatifloxacin						
A (a/d)	4/1	5/0	4/1	4/1	5/0	0/5
B (a/d)	5/0	4/1	4/1	4/1	0/5	2/3
C (a/d)	5/0	5/0	4/1	3/2	2/3	3/2
D (a/d)	5/0	4/1	4/1	3/2	2/3	2/3
% mortality	5	10	20	30	55	65

(A, B, C, D represents different experimental groups; a/d denotes number of alive (a) and dead (d) animals in each group)

Acute toxicity tests show rapid effects of drugs in the tested organisms in a short time to evaluate certain effects on the organism, such as mortality, luminescence, inhibition and immobility (Gueretz *et al*., 2017). *Daphnia magna* is a popular toxicity testing species (Lv *et al*., 2018). Several environmental organizations, including the American Society Organization for Testing and Materials (ASOTM), the US Environmental Protection Agency (USEPA), the International Organization for Standardization and the Organization for Economic Cooperation and Development have adopted standardized toxicity testing procedures using crustaceans (Ribeiro *et al*., 2018). Daphnids have become important model for system biology, prompting their use for prescreening toxicity testing before vertebrate testing (Siciliano *et al*., 2015). Most of toxicological studies with these crustaceans are based on acute toxicity

data for assessment of lethal concentrations (LC_{50}), for effective concentration (EC_{50}) and for immobilization (Bownik, 2017).

In our study the 48h-EC_{50} of cefixime and cefotaxime against *D. magna* was 77.92 mg/L and 25.82 mg/L. The ciprofloxacin EC_{50} for *V. fischeri*, *D. magna* and for *P. subcapitata* was reported to be 11.5 mg/L, 65.3 mg/L 3.9 mg/L respectively (Martins *et al*., 2012). In a study conducted by Andrieu *et al*. (2015), the calculated EC_{50} value of enrofloxacin and ciprofloxacin were 111.0 and 23.0 mg/L for chlorella and 69.0 and 71.0 mg/L for *M. macrocopa* respectively. Gonzalez-Pleiter *et al*. (2013) reported EC_{50} value for quinolone, levofloxacin and norfloxacin were 120, 80 and 3.31 mg/L on *P. subcapitata* and 0.4, 0.5, and 0.8 mg/L for *Anabaena sp.* respectively which was much lower than our reported EC_{50} value for gatifloxacin (330.8 mg/L) and gemifloxacin (489.42 mg/L). Dalla Bona *et al*. (2014) reported EC_{50} value of 4.3 mg/L (enrofloxacin) to 421 mg/L for sulfadimethoxine in *D. curvirostris* and from 6.2 mg/L (sulfaguanidine) to 312.2 mg/L (sulfaclozine) in *D. magna*. The benzimidazole resulted in lower EC_{50} value in *Daphnia magna* showing higher sensitivity of fresh water macro invertebrates (Bundschuh *et al*., 2016). The norfloxacin EC_{50} value was reported to be 141.3, 104.5 mg/L for *B. aeruginosa* respectively (Ge *et al*., 2020) and for tetracycline it was 94.4 mg/L for *Stentor coeruleus* and 40.1 mg/L for *Stylonychia lemnae* (Wang *et al*., 2020). The EC_{50} value reported for ciprofloxacin and enrofloxacin on *D. subspicatus was* 80 mg/L and 55 mg/L respectively (Ebert *et al*., 2011). Robinson *et al*. (2005) reported levofloxacin, clinafloxacin, enrofloxacin, ciprofloxacin, lomefloxacin, ofloxacin and flumequine as most toxic to model organisms during toxicity testing. In another study, the most sensitive organism observed was *M. aeruginosa* (5d EC_{50} ranging from 7.9 to 1960 mg/L), followed by *L. minor* (7d EC_{50} 53 to 2470 mg/L) and *P. subcapitata* (3 d EC_{50} 1100 to 22700 mg/L). Luo *et al*. (2018) reported the 48 h EC_{50} value of lomefloxacin in *D magna* was 473.7 µM. While Baumann *et al*. (2015) observed clarithromycin EC_{50} value of 2 mg/L for *Daphnia magna*. Wei *et al*., (2018) reported 96h EC_{50} value for chlorotetracycline, oxytetracycline, sulfadiazine, sulfamerazine, dibutyl phthalate, sulfamethazine to *Vibrio fischeri* were 6.67, 25.12, 67.61, 141.51, 148.38, 245.07 mg/L respectively. The 48 h EC_{50} value of flubendazole, fenbendazole and ivermectin for *D. magna* were reported to be 70.1, 16.7 and 0.59 µg/L respectively (Bundschuh

et al., 2015). Similarly the 48h treatment period, the EC_{50} values for flubendazole and fenbendazole in *D. magna* has been reported to be 0.0448 and 0.0193 respectively (Puckowski *et al*., 2017). The EC_{50} values for *D. subspictaus* were 5.56 and 8.0 mg/L for enrofloxacin and ciprofloxacin (Ebert *et al*., 2011). Nie *et al*. (2009) reported EC_{50} value of norfloxacin was 38.49 mg/L for *Scenedesms obliquus*. Peres *et al*. (2012) reported EC_{50} value of ofloxacin as 8.22 µg/L to *E. coli*. In the study of Van der Grinten *et al*. (2010) the toxicity of ciprofloxacin to *D. magna* was investigated and the observed EC_{50} value observed was 190 mg/L. Zounkova *et al*. (2011) studied toxicity of antibiotics including flumequine using six model organisms. The EC_{50} value range for flumine and oxytetracycline on *P. putida* was reported between 0.22 mg/L to 86 mg/L and for *D. magna* showed 400 mg/L.

In the study of Grinten *et al*. (2010) the observed EC_{50} value observed was 190 mg/L. Ebert *et al*. (2011) reported the EC_{50} values for cyanobacterium were 173, 10.2 mg/L, for *Lemna minor* was 107 and 62.5 mg/L and for *D. subspictaus* were 5565 and 8042 mg/L for enrofloxacin and ciprofloxacin respectively. The EC_{50} value reported by Andrieu *et al*. (2014) for enrofloxacin and ciprofloxacin for *Chorella sp.* and *M. macrocopa* were 111, 23.0 mg/L and 69.0 and 71.0 mg/L. The benzimidazole resulted in lower EC_{50} value in *Daphnia magna* showing higher sensitivity of fresh water macro invertebrates (Bundschuh *et al*., 2015). Ribeiro *et al*. (2018) reported 48h-EC_{50} value of ceftiofur was 139 µM for *D. magna*. In the bacterial plate test the EC_{50} value for Flumequine was 0.2 mg/L (Grinten *et al*., 2010). The EC_{50} value of chlortetracycline, oxytetracycline, sulfadiazine, sulfamethazine and sulfamerazine on *V. fischeria* are found to be 25.12, 6.67, 245.07 67.61, 141.51 mg/L respectively (Wei *et al*., 2018). Havelkova *et al*. (2016) observed toxic effects of penicillin G, vancomycin and tetracycline on *P. subcapitata*, *D. magna* and *V. fischeria*. Tetracycline showed highest toxicity to *P. subcapitata* (72h-EC_{50} 1.82 mg/L) and *D. magna* (48h-EC_{50} 8.16 mg/L).

6.3. Chronic toxic effects of cephalosporin (cefixime and cefotaxime) and fluoroquinolone (gemifloxacin and gatifloxacin) antibiotics to *D. magna*

The toxic effects of two classes of antibiotics were studied on *D. magna*. Adverse chronic effect of antibiotics were observed on daphnids during 21 days exposure. The

reproductive indicators: survival of neonates, first day of brood, number of young per female and number of young per brood were measured. The results for chronic toxic effect of cefixime on *D. magna* are shown in **Fig. 3**. The survival of *D. magna* was not significantly inhibited when exposed upto 2.1 mg/L and survival was observed to be 85% at the highest antibiotic concentration (56.7 mg/L) (**Fig. 3a**). No difference observed in the first day of brood and control (**Fig. 3b**). The number of young per female/brood were not affected with the increase in cefixime antibiotic concentration in the chronic assay (**Fig. 3c, 3d**).

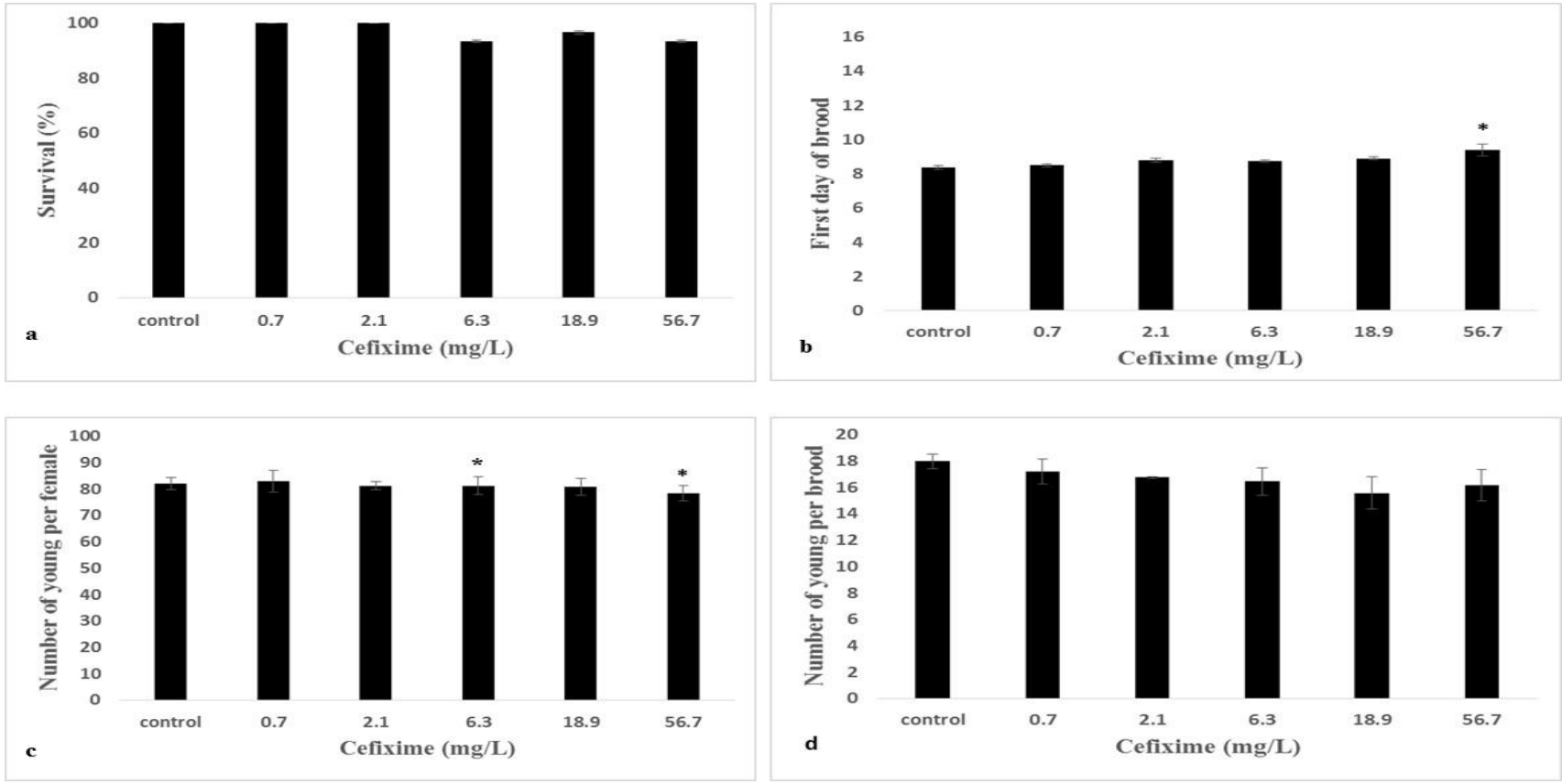


Fig. 3: Results of 21 d chronic exposure to cefixime in *D. magna*. Error bars correspond to standard error and * represents statistically significant differences (Tukey test, $p \leq 0.05$) between the different cefixime concentrations and the control.

The toxic effects of cefotaxime antibiotic on *D. magna* are presented in **Fig. 4**. With the increase in antibiotic concentration, significant decrease in daphnid survival was observed and 50% daphnids survived at the highest concentration (16.2 mg/L) of cefotaxime (**Fig. 4a**). Delay in reproduction at concentrations higher than 5.4 mg/L was observed (**Fig. 4b**), and the significant decrease in number of young per female were also observed after 0.60 mg/L antibiotic concentration. The number of young ones per brood were also significantly decreasing after 1.8 mg/L cefotaxime concentration in the chronic assay (**Fig. 4c, 4d**).

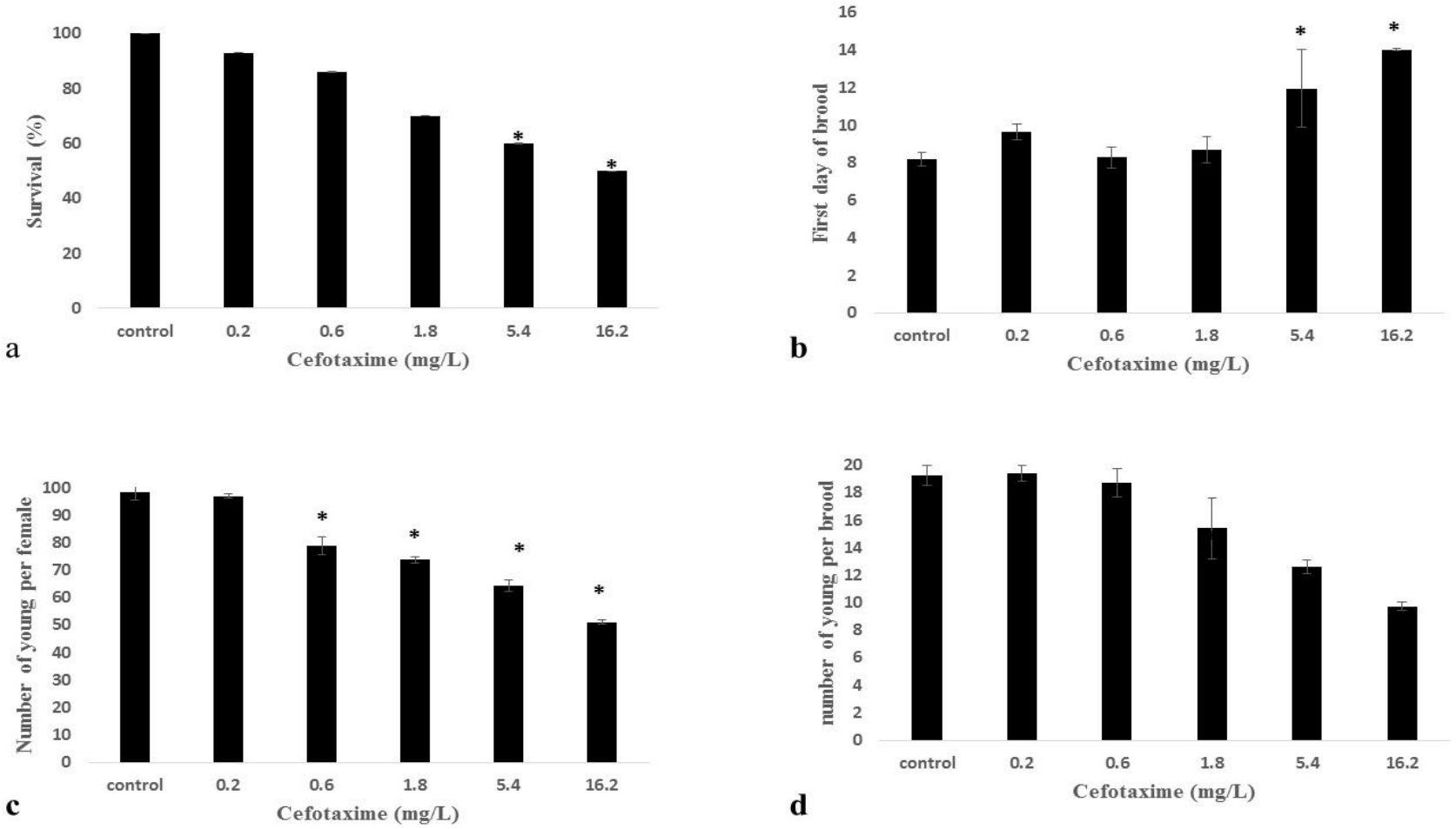


Fig. 4: Results of 21 d chronic exposure to cefotaxime in *D. magna*. Error bars correspond to standard error and * represents statistically significant differences (Tukey test, $p \leq 0.05$) between the different cefotaxime concentrations and the control.

The chronic effect of gemifloxacin was observed on *Daphnia magna*. Survival of daphnids were decreasing in a dose dependent manner. All daphnids die after 94.5 mg/L concentration of gemifloxacin (**Fig. 5a**). The first day of reproduction was statistically significant till 10.5 mg/L and after 31.5 mg/L the toxic effect of the antibiotic concentration could not be tested since all daphnids die at these concentrations (**Fig.** 5**b**). The average number of neonates per female was constantly decreasing with the increase in concentration when compared with control (**Fig. 5c**). The number of young ones per brood decreased after 10.5 mg/L (**Fig. 5d**). Even noteworthy effects of antibiotic concentrations were observed, it is doubtful that the same results will be observed in the environment because the concentrations found in nature are below our tested range of mg/L and may not negatively affect aquatic organisms (Allyson *et al*., 2020).

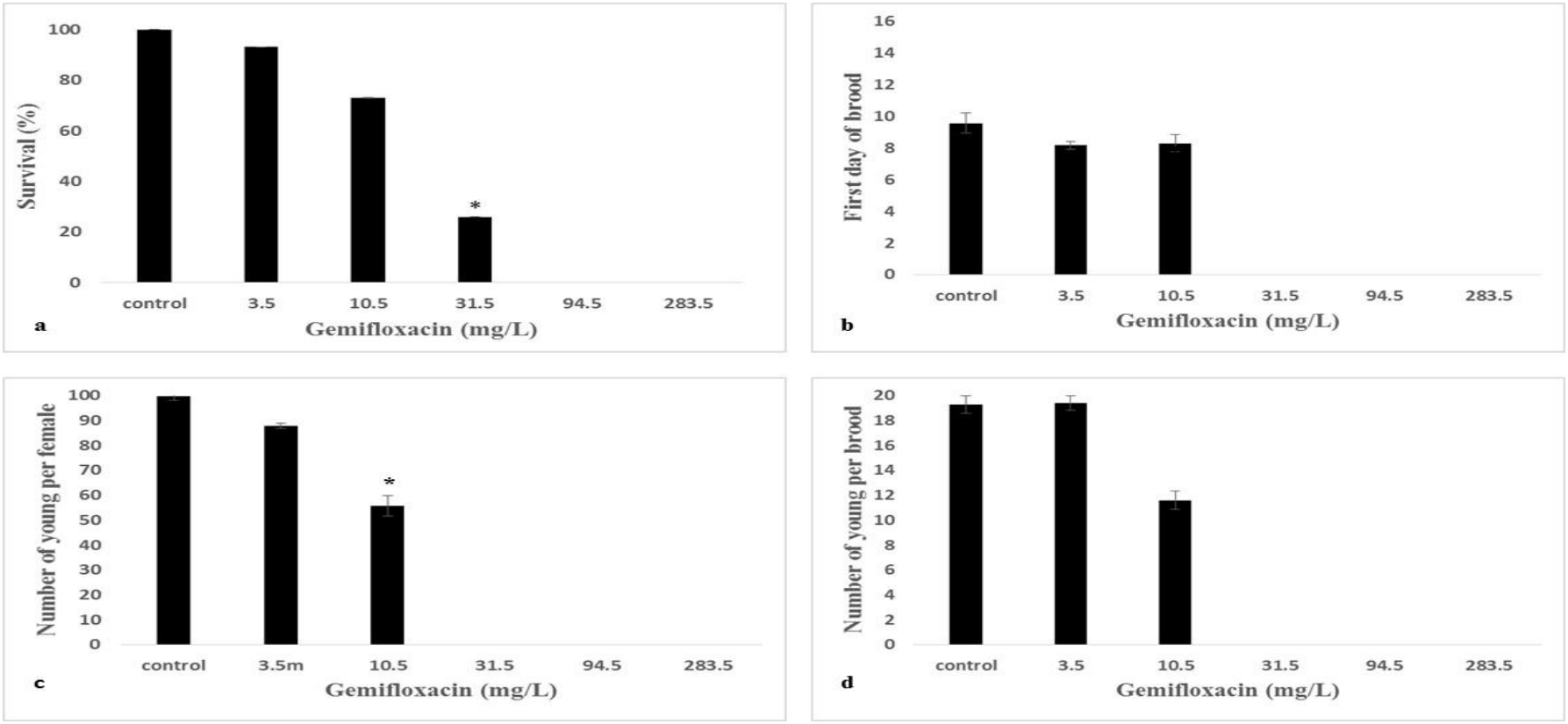


Fig. 5: Results of 21 d chronic exposure to gemifloxacin in *D. magna*. Error bars correspond to standard error and * represents statistically significant differences (Tukey test, $p \leq 0.05$) between the different gemifloxacin concentrations and the control

The toxic effects of gatifloxacin antibiotic on *D. magna* causes adverse effects during 21 days of exposure **(Fig. 6)**. The survival of daphnids reduced in a dose dependent manner (**Fig 6a**). At gatifloxacin antibiotic concentration i.e. 202.5 mg/L, 100% mortality was observed. The initial concentrations of gatifloxacin had little effect at first day of reproduction and after 67.5 mg/L the toxic effect of the antibiotic concentration could not be tested since all daphnids die at these concentrations (**Fig 6b**). Comparing with control the average number of young per female were also affected and at highest concentrations (67.5 and 202.5 mg/L) and no production of young ones were observed (**Fig. 6c**). Significant decrease in number of young per brood was observed at 22.5 mg/L and no daphnid was observed after 67.5 mg/L gatifloxacin concentration (**Fig. 6d**).

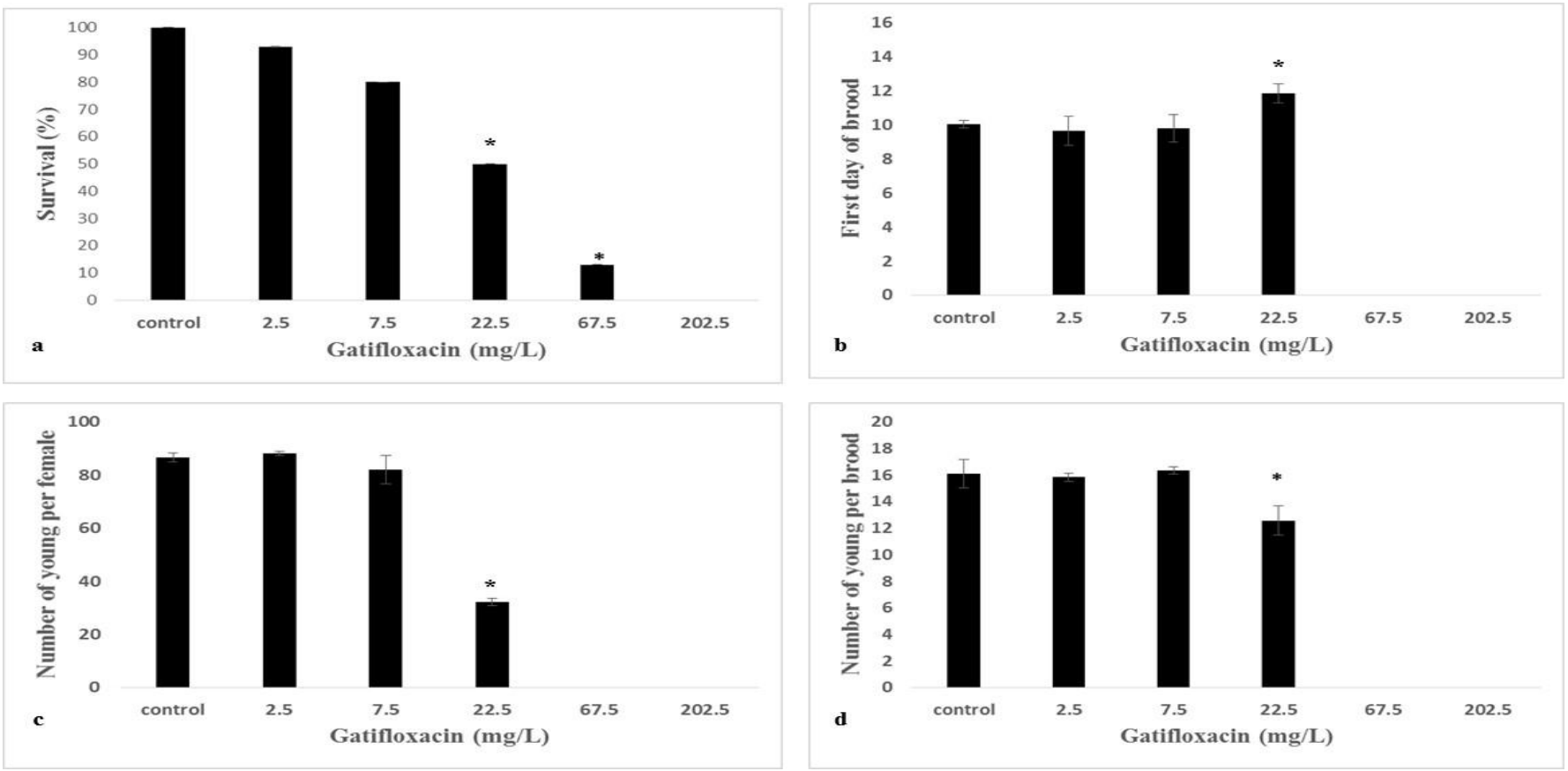


Fig. 6: Results of 21 d chronic exposure to gatifloxacin in *D. magna*. Error bars correspond to standard error and * represents statistically significant differences (Tukey test, $p \leq 0.05$) between the different gatifloxacin concentrations and the control.

Chronic toxicity testing is used to describe specific organs affected after repeated administration and to characterize dose-response relationships. The life cycle parameters of daphnia are well studied. Dose-response data predicts the effects of exposure at other dose levels and life stages (Stollewerk, 2010). Since antibiotics target specific pathways in humans, they may affect the same in aquatic non target organisms having the drug target homologies (Siciliano *et al*., 2015).

In our study cefixime antibiotic show no significant inhibition of *D. magna* and survival was observed to be 85% at highest antibiotic concentration (56.7 mg/L). There was no significant difference observed in the first day of brood and control. The number of young per female and number of young ones per brood were not affected with the increase in cefixime antibiotic concentration. The results were supported by Bernot *et al*. (2005) were they have shown that emidizolen based ionic liquid cause significant reduction in first brood neonates and average neonates and also studied that such reduction in reproduction output of populations could cascade through natural fresh water ecosystems. In another study Bringolf *et al*. (2010) reported fluoxetine disrupt several aspects of reproductive parameters in fresh water mussel. Quinolones negatively and significantly affect the life history parameters reducing survival of *D. magna* below 80% at concentration higher than 2.5 mg/L for enrofloxacin, ciprofloxacin, norfloxacin and moxifloxacin (Kergaravat *et al*., 2020). Kim *et al*. (2017) reported the chronic toxic effect of cefadroxil and cefradine on the growth and reproductive parameters of *D. magna*. Martins *et al*. (2012) reported similar chronic toxicity of ciprofloxacin on *D. magna* and *P. subcapitata*. The affected parameter were the somatic growth rate, number of neonates per brood, intrinsic rate of population, increase fecundity of females, number of broods per female. The *P. australis* were exposed to five different concentrations of ciprofloxacin, oxytetracycline and sulfamethazine for toxicity bioassay. Antibiotic concentrations above 10 mg/L had toxic effects on leaf chlorophyll, root activity and induce hormesis (Liu *et al*., 2013). Adermei *et al*. (2018) exposed erythromycin, clarithromycin, sulfathiazole, ciprofloxacin to *R. subcapitata.* They showed significant growth inhibition at higher concentration.

In our study cefotaxime antibiotic significantly reduce survival of daphnids and 50% mortality of daphnids were observed at 16.2 mg/L antibiotic concentration. Delay in first day of reproduction at concentrations higher than 5.4 mg/L and young per female after 0.60 mg/L antibiotic concentration. The number of young ones per brood was

also significantly decreasing after 1.8 mg/L. With the increase in antibiotic concentration the first day of reproduction was prolonged, which may be due to antibiotics delaying the rate of oogenesis. Deliguoro *et al*. (2019) showed similar effect of flumequine antibiotic on survival, growth and reproduction of *D. magna.* Flaherty and Dodson, (2005) reported significant effect of fluoxetine exposure on daphnia fecundity. According to the study by Luo *et al*. (2018), exposure of *D. magna* to lomeofloxacin caused a significant death of adults. Baumann *et al.* (2015) reported no acute toxicity at the highest clarithromycin concentration and reproduction was observed to be inhibited by metabolite N-desmethyl-clarithromycin. Dalla Bona *et al*. (2015) observed mortality rate of 100 and 50% in Fo generation at enrofloxacin and trimethoprim concentration of 13 and 50 mg/L respectively. And in F_1 generation a deteriorating trend of the response with enrofloxacin and ciprofloxacin was observed both for growth and reproduction. Pan *et al*. (2017) reported the toxic effect of norfloxacin on mortality, heart rate and behavior of *D. magna*. Han *et al*. (2016) reported trimethoprim growth retardation in *Tigriopus japonicas* in dose dependent manner, fecundity showed deline at the highest dose and effect on population reproductive system was observed which effects individual and population growth.

In our study all daphnids die after 94.5 and 202.5 mg/L concentration of gemifloxacin and gatifloxacin respectively. The number of young ones per brood decreased after 10.5 and 22.5 mg/L concentration of gemifloxacin and gatifloxacin respectively. The study by Luo *et al*. (2018) performed with Daphnids exposed to lomefloxacin antibiotic, demonstrated significant inhibitory effect on survival, delay in the time of first brood and increase in body length. The toxicity of penicillin antibiotic on *D. magna* exposed to different concentrations (11.79, 117 and 1179 mg/L) for 2 and 24 h. No daphnia mortality was observed but effect on swimming activity and physiological parameters were reported at all concentrations (Bownik *et al*., 2019).

6.4. Toxicity of *Danio rerio*

Conserved vertebrate biology, transparent embryos, rapid development and high fecundity are some of the main attractions of *Danio rerio* for toxicity studies. Newly fertilized zebra fish embryos were treated with antibiotics for 96 hrs. Every after 24 hrs embryos were observed for apical observations such as coagulated embryos, non-

detachment of tail, lack of somite formation and mortality percentage. Any positive outcome in one of these observations mean that the zebra fish embryos is dead.

6.4.1. Acute toxicity of cephalosporin (cefixime and cefotaxime) antibiotics on *Danio rerio*.

The mortality in each test concentrations were observed and recorded at 24, 48, 72 and 96 hrs of experiment. The survival of embryos were observed 100% in all concentrations of cefixime and cefotaxime antibiotics. 0% mortality was observed in negative control and internal plate control (**Table 3, 4**). There were no lethal effects and other malformations recorded in embryo development (**Fig. 7, 8)**. The exposure of embryos to cefixime and cefotaxime concentration up to 100 µg/ml did not cause any mortality within 96 hrs of exposure and they were found to be safe under the tested experimental conditions in the present study.

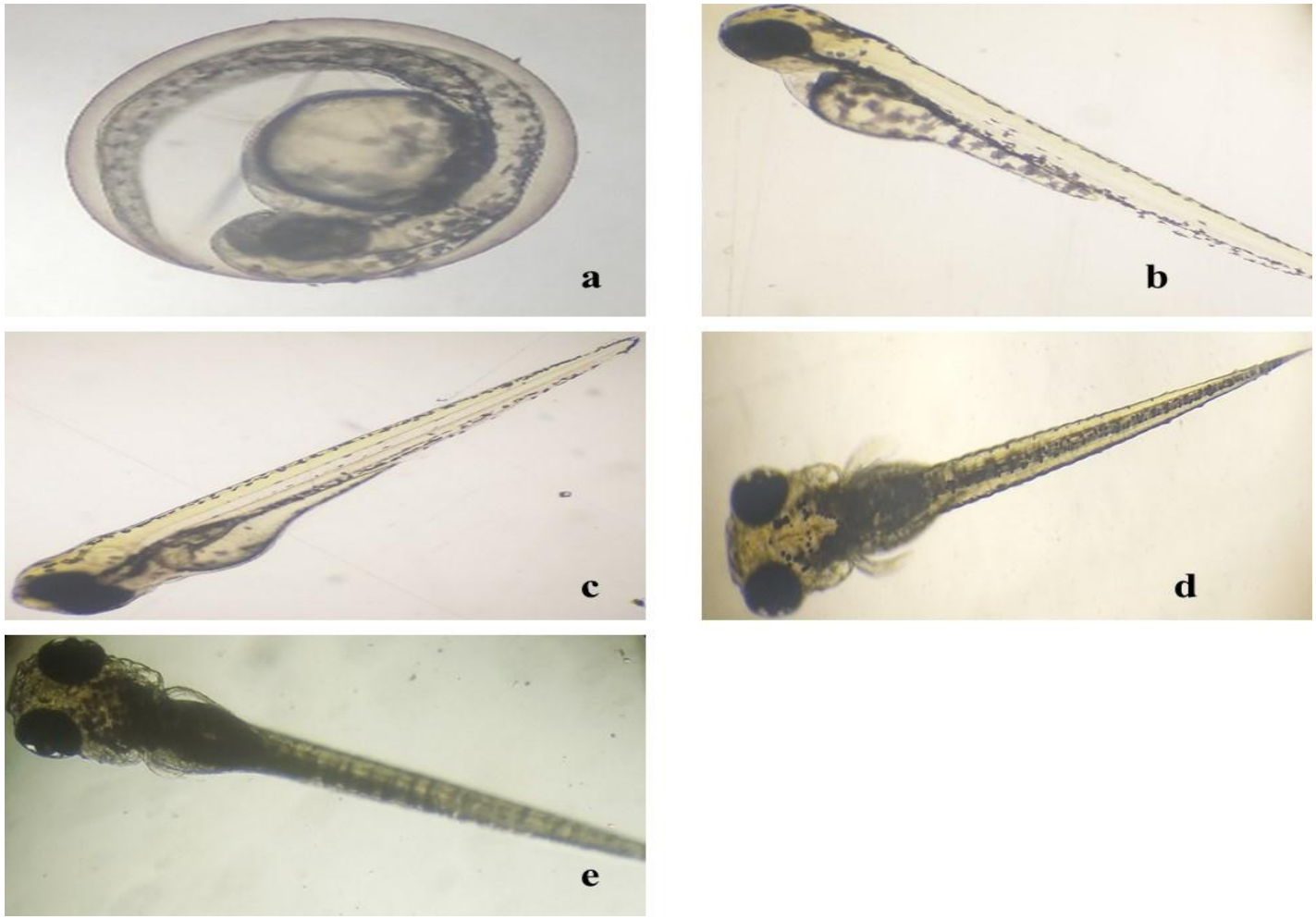


Fig. 7: Cefixime antibiotic treated zebra fish embryos after; a (24 hr), b (48 hr), c (72 hr), d (96 hr), e (control)

Table 3: Apical observations of cefixime acute toxicity in zebra fish embryo

Cefixime concentrations (µg/ml)							
Observations	**100**	**25**	**12.5**	**6.25**	**3.125**	**Negative control group**	**Internal plate control**
Coagulated embryos	0%	0%	0%	0%	0%	0%	0%
Lack of somite formation	0%	0%	0%	0%	0%	0%	0%
Non-detachment of tail	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Other phenotypic abnormalities	0%	0%	0%	0%	0%	0%	0%
Mortality %	0%	0%	0%	0%	0%	0%	0%

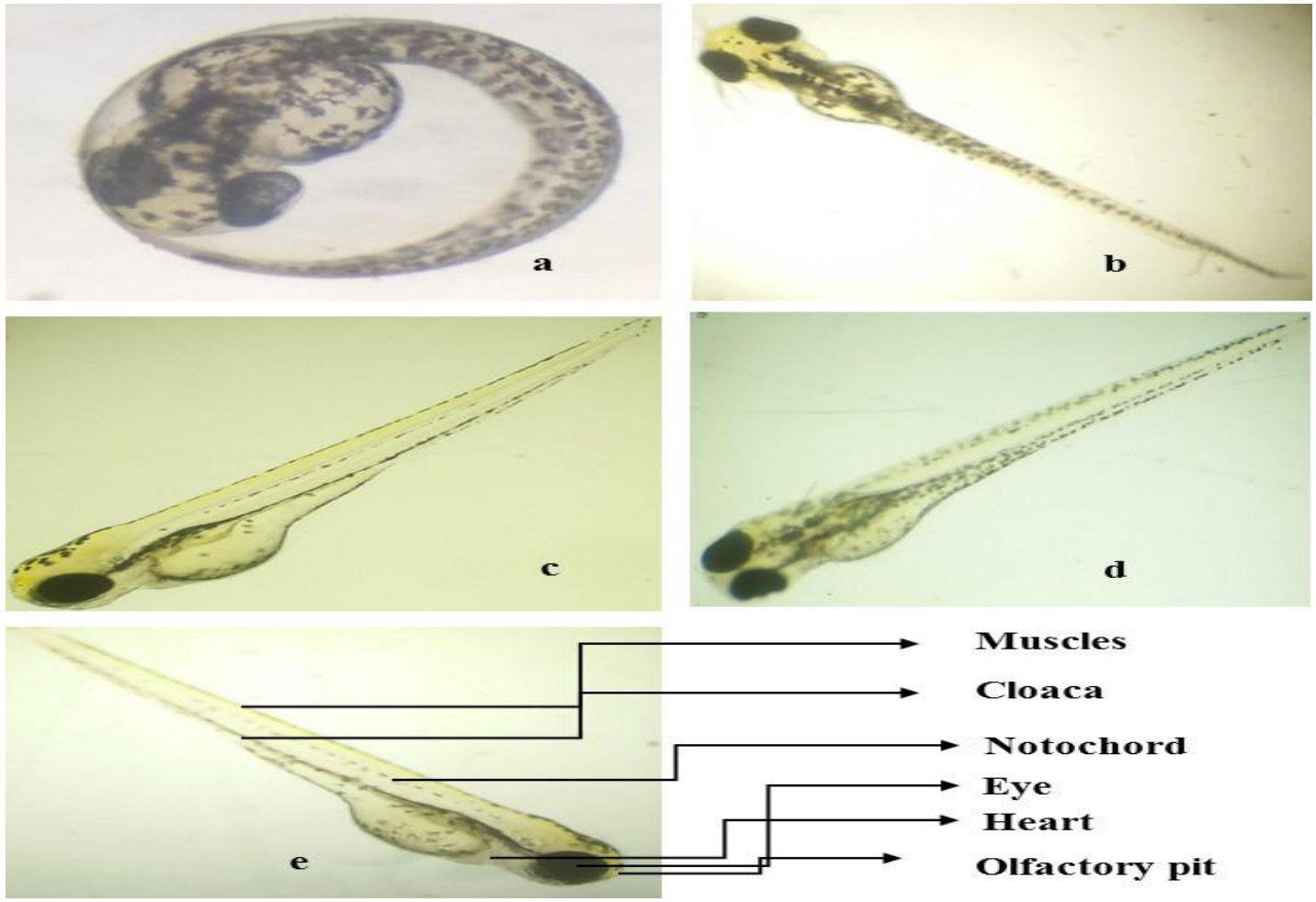


Fig. 8: Cefotaxime antibiotic treated zebra fish embryos after; a (24 hr), b (48 hr), c (72 hr), d (96 hr), e (control)

Table 4: Apical observations of cefotaxime acute toxicity in zebra fish embryo

Cefotaxime concentrations (µg/ml)							
Observations	**100**	**25**	**12.5**	**6.25**	**3.125**	**Negative control group**	**Internal plate control**
Coagulated embryos	0%	0%	0%	0%	0%	0%	0%
Lack of somite formation	0%	0%	0%	0%	0%	0%	0%
Non-detachment of tail	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Other phenotypic abnormalities	0%	0%	0%	0%	0%	0%	0%
Mortality %	0%	0%	0%	0%	0%	0%	0%

6.4.2. Acute toxicity of fluoroquinolone (gemifloxacin, gatifloxacin) antibiotics on *Danio rerio*.

No effects of categorical end points were recorded by gemifloxacin and gatifloxacin antibiotics (**Fig. 9, 10)** until 96 hrs. The survival of embryos were observed to be 100% for the control and treatment groups. No lethal and other malformations were recorded in embryo development (**Table 5, 6**). Thus the exposure of embryos to gemifloxacin and gatifloxacin up to 100 µg/ml concentration did not cause any mortality within 96 hrs of exposure and they were found safe under the tested experimental conditions in the present study.

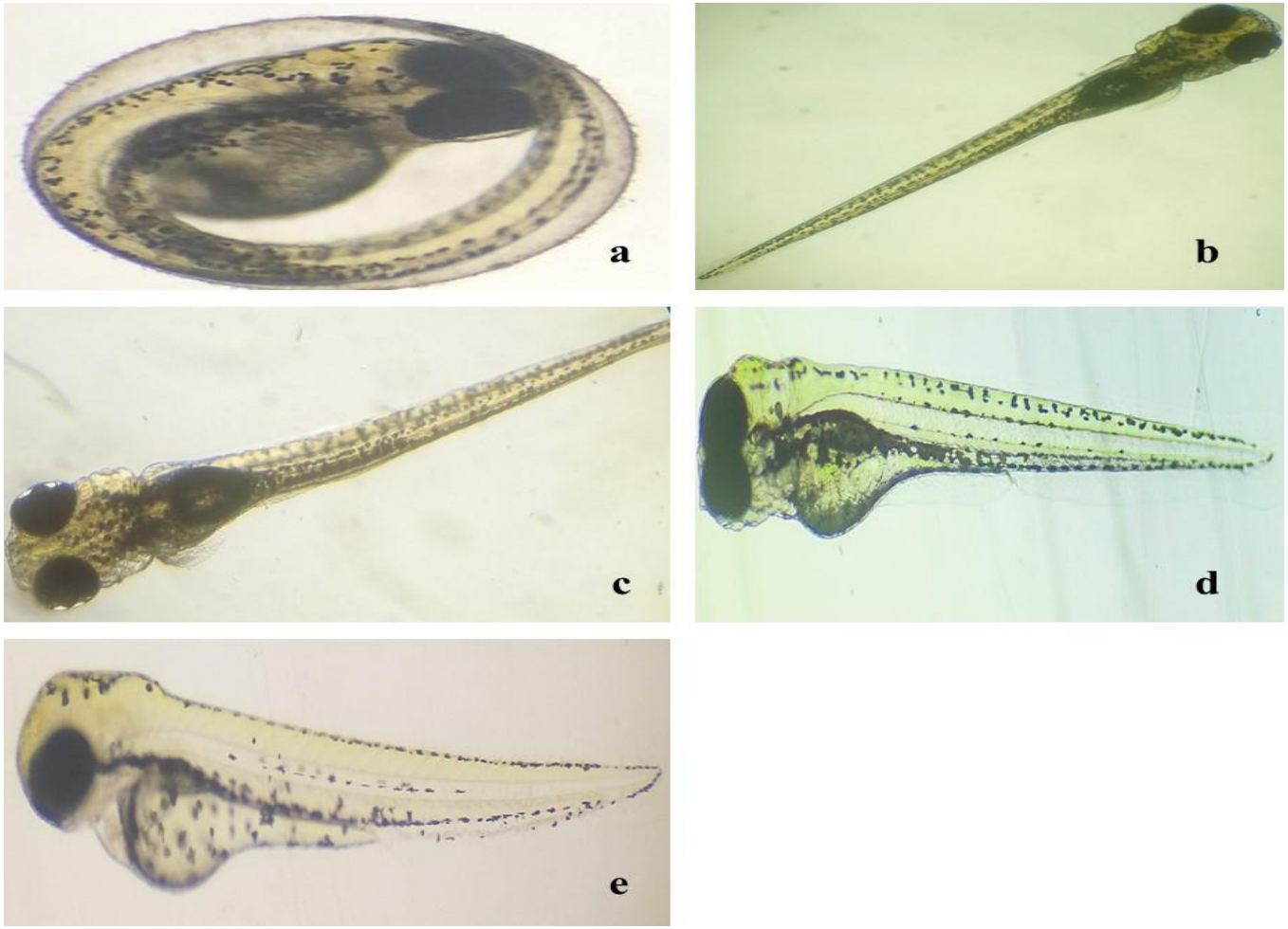


Fig. 9: Gemifloxacin antibiotic treated zebra fish embryos after; a (24 hr), b (48 hr), c (72 hr), d (96 hr), e (control)

Table 5: Apical observations of gemifloxacin acute toxicity in zebra fish embryo

Gemifloxacin concentrations (µg/ml)							
Observations	**100**	**25**	**12.5**	**6.25**	**3.125**	**Negative control group**	**Internal plate control**
Coagulated embryos	0%	0%	0%	0%	0%	0%	0%
Lack of somite formation	0%	0%	0%	0%	0%	0%	0%
Non-detachment of tail	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Other phenotypic abnormalities	0%	0%	0%	0%	0%	0%	0%
Mortality %	0%	0%	0%	0%	0%	0%	0%

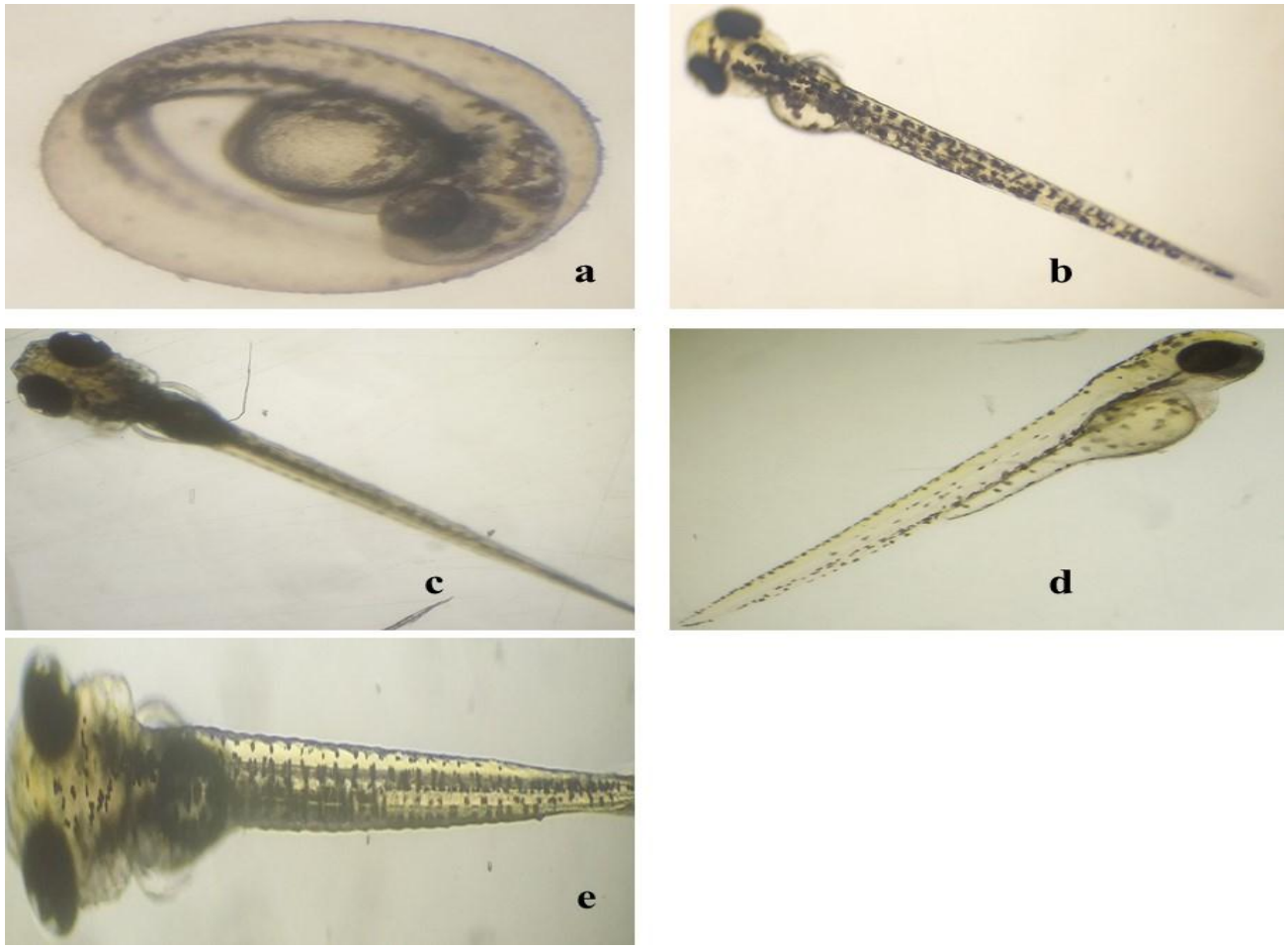


Fig. 10: Gatifloxacin antibiotic treated zebra fish embryos after; a (24 hr), b (48 hr), c (72 hr), d (96 hr), e (control)

Table 6: Apical observations of gatifloxacin acute toxicity in zebra fish embryo

Gatifloxacin concentrations (µg/ml)							
Observations	**100**	**25**	**12.5**	**6.25**	**3.125**	**Negative control group**	**Internal plate control**
Coagulated embryos	0%	0%	0%	0%	0%	0%	0%
Lack of somite formation	0%	0%	0%	0%	0%	0%	0%
Non-detachment of tail	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Other phenotypic abnormalities	0%	0%	0%	0%	0%	0%	0%
Mortality %	0%	0%	0%	0%	0%	0%	0%

Zebra fish played an important role in ecotoxicity and developmental studies due to the presence of transparent embryos (Bootorabi *et al*., 2017). Previous studies showed zebra fish embryos act as the good indicators of developmental toxicity by toxicant stress. For instance Yan *et al*, (2018) reported toxic effect of sulfamethazine on *Danio rerio* embryos, when exposed upto 200 mg/L. The results showed coagulated malformations, spinal curvature and edema. Under microscope coagulated embryos can be determined after 24 hrs and they appear dark under the microscope. Normally developed embryos show spontaneous movements, indicates formation of somite which could be recorded after 24 hrs. The tail detechment from yolk observed after 24 hrs and the body length are also significant indicator of growth, which may be induced by loss of nutrients. The rate of heart beat can also be observed after 48 hrs in developing zebra fish embryos. Hatching is the result of biochemical, physical and osmotic mechanisms and delayed hatching induce death of embryos (Osman *et al*., 2007). Our results indicated no developmental deformalities on zebra fish embryos upto the highest concentration of cephalosporin and fluoroquinolone antibiotics till 96 hrs. The results were in agreement with the previous studies by Zhang *et al*. (2014), who reported 72 h EC_{50} value for fluoroquinolone to zebra fish embryo to be 481.3

mg/L and more than 90% of embryos were hatched when compared with control. The enrofloxacin and danofloxacin antibiotics were tested using zebra fish embryos did not cause any effect upto 1 mg/L (Carlsson *et al*., 2013). Amoxacillin and oxytetracycline showed no toxic effect below 100 mg/L on Zebra fish (Oliveira *et al*., 2013). The results by Plhalova *et al*. (2014) also showed no significant pathological changes observed in organs of fish exposed to different ciprofloxacin concentration.

The results by other researchers reported developmental deformailities in zebra fish embryos. The results by Qui *et al*. (2020) showed single and joint toxic effect of sulfamonomethoxine, tetracycline, cefotaxime sodium and enrofloxacin antibiotics on zebra fish. At 100 μg/L the body length of zebra fish larva were significantly shortened. Zhang *et al*. (2015) reported tetracycline exposure induce shortened body length, delay in hatching and increased yolk sac. Wang *et al*. (2020) results showed 96 h EC_{50} value 8.57 mg/L, induced zebra fish embryo mortality, decreased heart rate and hatching rate. Numerous other studies have reported toxic effects of antibiotics on zebra fish embryos, where concentration of antibiotics were used higher than our selected concentration. The study by Chen *et al*. (2017) demonstrated deformity in body length and embryonic notochord twisted when zebra fish embryos were exposed to cefazolin antibiotics. Ding *et al*. (2017) reported 72h-EC_{50} value of 130.3 mg/L for hatching rate and for 120h-EC_{50} value of 135.1 mg/L for malformation rate of zebra fish embryos. Xi *et al*. (2019) reported the significant toxic effect of norfloxacin which results in embryonic mortality and malformation of the zebra dish embryos. Yan *et al*. (2016) also demonstrated that exposure to the lower concentration of norfloxacin and sulfamethoxazole (200 μg/L) effects survival, body weight, hatching and egg production of zebra fish. Peltzer *et al*. (2017) reported significant effect on larval development, growth rate of *R. arenarum* exposed to enrofloxacin and ciprofloxacin antibiotics. Han *et al*. (2018) results showed cephalosporin toxicity on zebrafish embryonic development as well as alter the expression of various genes. Zhang *et al*. (2010) reported teratogenic effect of cefazedone and cefazolin sodium antibiotics on zebra fish embryos. Tetracycline exposure of embryos resulted in delayed hatching, shorter body length and uninflated swim bladder (Zhang *et al*., 2015). Zhang *et al*. (2013) results suggested cephalosporin functional groups toxicity

were involved in abnormal organogenesis including cardiovascular development, notochord, cranial nerve and pigment formation. Zhang *et al*. (2014) found that ceftazidine and cefotaxime (15 and 10 mg/L) showed toxic effects on zebra fish larvae including mild blood pooling, abnormal abdomens, short and congested bodies. Shen *et al*. (2019) observed mortality increase with the increase of gatifloxacin and ciprofloxacin antibiotics. Gatifloxacin induce pericardial edema and ciprofloxacin did not induce any morphological abnormality. The acute toxic effect of maduramicin at 10 mg/L results in death of zebra fish. The sub lethal effects at 2.5 mg/L include damage in liver, gills and intestine of zebra fish after 14 days of exposure (Ni *et al*., 2019). Wang *et al*. (2017) observed zebra fish death rate 66.0% at 50 mg/L of tetracycline antibiotic. Shi *et al*. (2019) observed reduced body length, delayed hatching, malformations, when zebra fish embryos were treated with triclocarban. Qian *et al*. (2017) reported abnormalities including small head, eyes, spherical abdomen and pericardial edema when zebra fish were treated with cefatirizine and its impurities. Liang *et al*. (2019) observed mortality of zebra fish embryos increased significantly and hatching rate decreased at 25 mg/L norfloxacin concentration. Exposure to pharmaceutical mixture (carbamazepine, acetaminophen, venlafaxine and gemfibrozil) caused a significant decline in embryo production, viability of embryo produced and atretic oocytes (Galus *et al*., 2013). The heart rate showed biphasic distribution after 2 day post fertilization when zebra fish embryos were exposed to azithromycin, tilmicosin, clarithromycin and tylosin (Yan *et al*., 2018).

6.5. Characterization of adsorbent materials

6.5.1. FTIR analysis

The FT-IR spectra of the prepared beads were recorded at room temperature within the range of 4000-450 cm^{-1} and obtained spectra are shown in **Fig. 11**. The presence of N-H, O-H and CO groups are considered as a good choice for the adsorption process (Kumar *et al*., 2017). The C-H stretching vibration can be seen between 3700-3800 cm^{-1}, which suggested the presence of alkenes in AWC (a), CAW (b) and WAC (c) beads. The strong adsorption bands at 3273 cm^{-1}, 3255 cm^{-1} and 3288 cm^{-1} on AWC, CAW and WAC beads may be attributed to the symmetrical stretching vibrations of -OH group. The characteristic peaks observed at 2359 cm^{-1}, 2362 cm^{-1}

and 2355 cm^{-1} are due to the presence of N-H group. The reaction between aldehyde groups and some amino groups results in the formation of amine groups. And this transformation is supported by the presence of peaks at 1643 cm^{-1}, 1647 cm^{-1} and 1649 cm^{-1}, highlights a C=O stretch suggestive of alkenes.

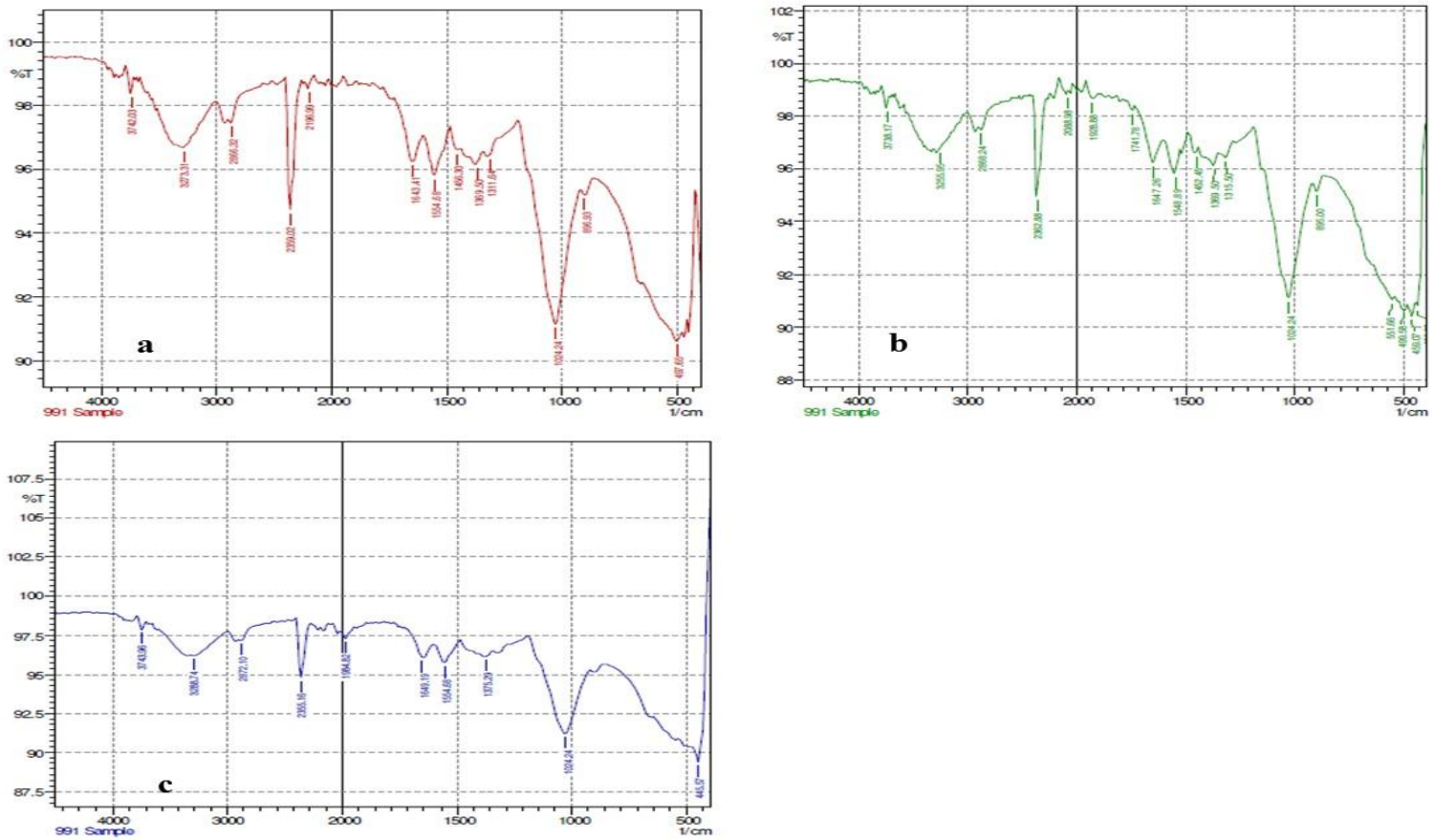


Fig. 11: FTIR images of beads (a) AWC, (b) CAW and (c) WAC

6.5.2. SEM analysis

Scanning electron micrographs (SEM) presents the morphological characters and surface texture of the beads (before and after adsorption). The pre-processed SEM image of AWC beads (**Fig. 12a)** illustrates topographical features of being irregular to wavy in appearance substantiating the fact that the material under question is definitely multiple layered. The individual surface of aggregates seems to be somewhat smooth with definitive and refractile margins. Overall the material seems to be more or less in unison, shows different gradients of density along with presence of sparsely scattered fissures and occasional pores. The CAW beads (**Fig. 12b**) shows surface topography to be multiple layered and uneven along with formation of chunks of varying sizes. The chunks are widespread, bit smooth somewhere and more or less give a cauliflower like appearance with inconsistent margins. The presence of fissures and conspicuous pores can be also seen selectively. The WAC bead (**Fig. 12c**) surface seems multiple layered, rough, scattered in patterns of being sparse, discrete to be assorted somewhere thus giving an uneven outlook. The inhomogeneous material shows varying grades of densities along with formation of distant pores.

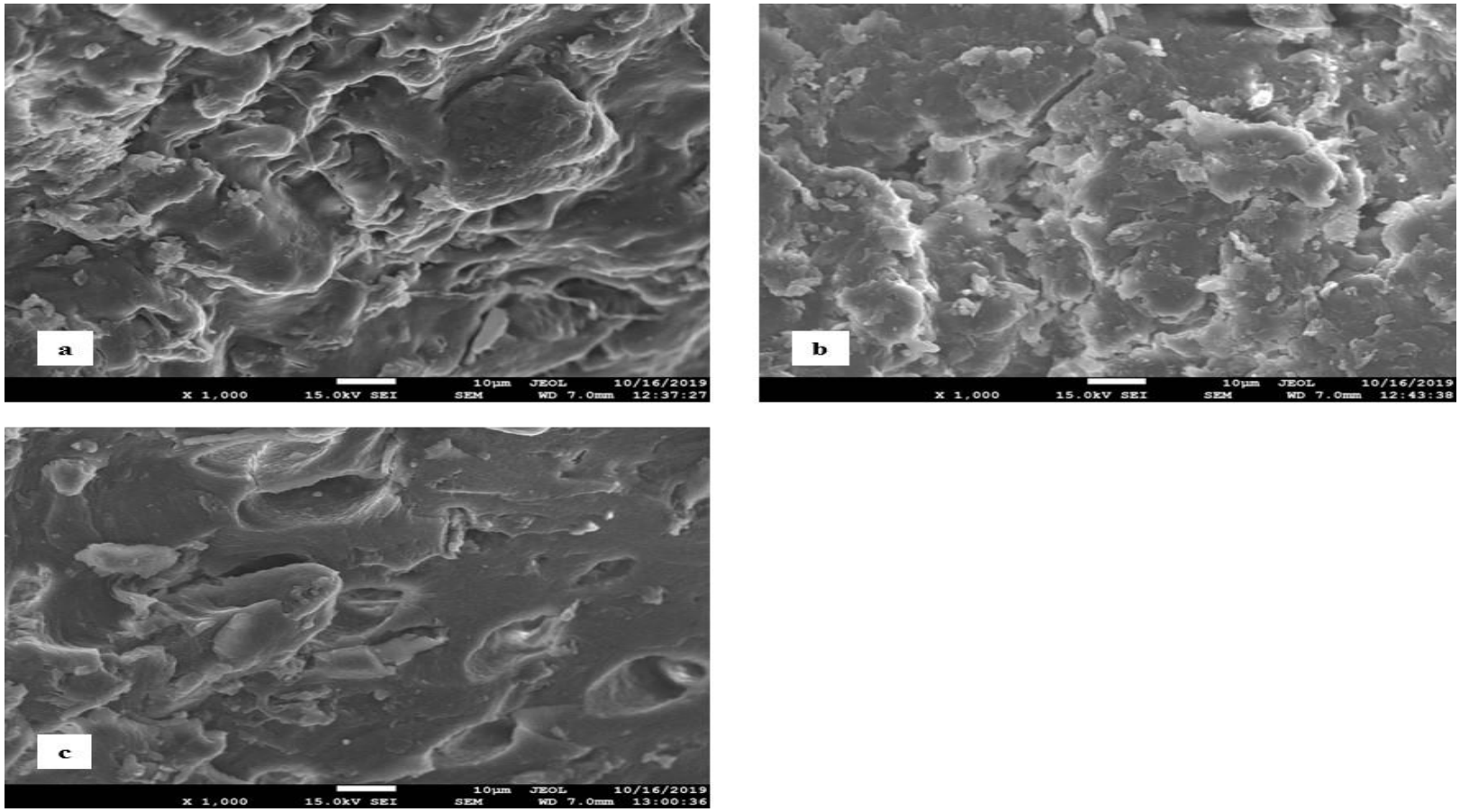
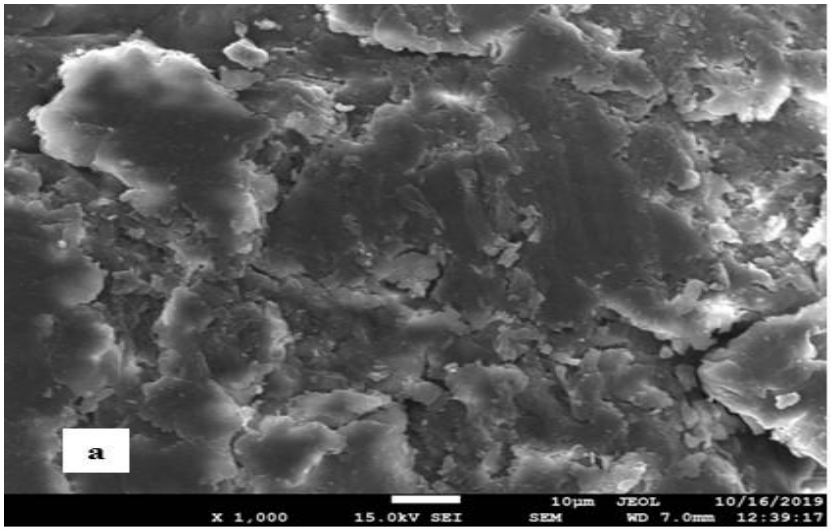


Fig. 12: SEM micrograph images of beads before adsorption (a) AWC (c) CAW and (c) WAC

The images of beads after adsorption (**Fig. 13 a-d**) clearly shows a reorganization in arrangement and composition. The agglomerates have become more concentrated, coerced thus giving rise to noticeable fissures. The material can be seen presenting more of a clumpy to somewhat discrete appearance i.e. ranging from 1µm–40 µm thus clearly indicating the difference in density and homogeneity. The topography still presents to be multiple layered but individually aggregates presents more of an even surface topography (when compared to pre-processed image). The SEM images of CAW beads in post processed images (**Fig. 13 e-h**) shows material to be multiple layered along with a bit refractive somewhere, in homogenous and becoming more condensed in composition. The post processed image shows that the agglomerates previously formed have mellowed down in terms of size and adhesion pattern thereby clearly indicating an activity. The average size of agglomerates when compared changed from being as large as 20µm (preprocessed image) to being reduced to 5µm (post processed image) thus clearly indicating a modification. The post processed SEM image (**Fig. 13 i-l**) after adsorption clearly shows that it has resulted due to some chemical processes. The agglomerates have further regained their size, become more condensed, tightly packed thereby look more coerced, though the surface seems to be still rough, multiple layered but overall seems to be more fortified.


a
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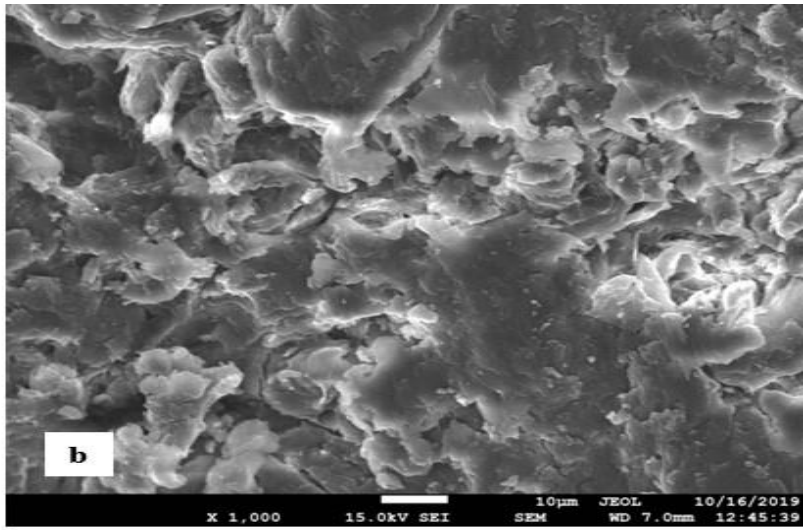
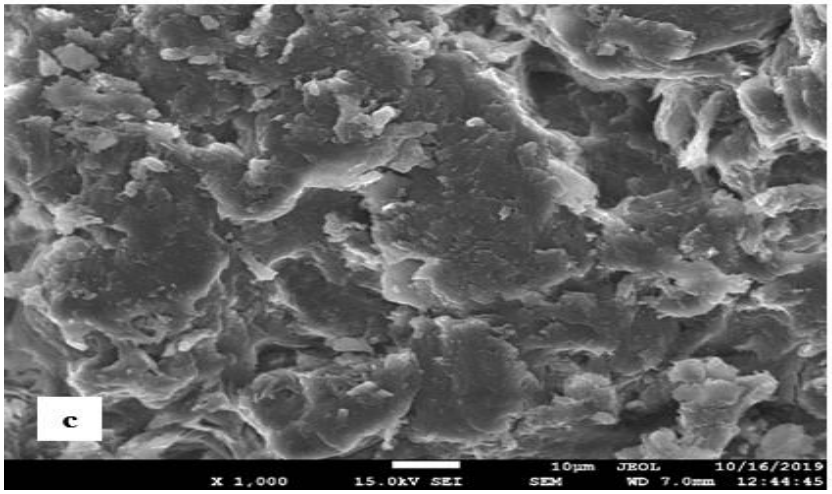

b
X 1,000 15.0kV SEI 10μm JEOL SEM WD 7.0mm 10/16/2019 12:45:39


c
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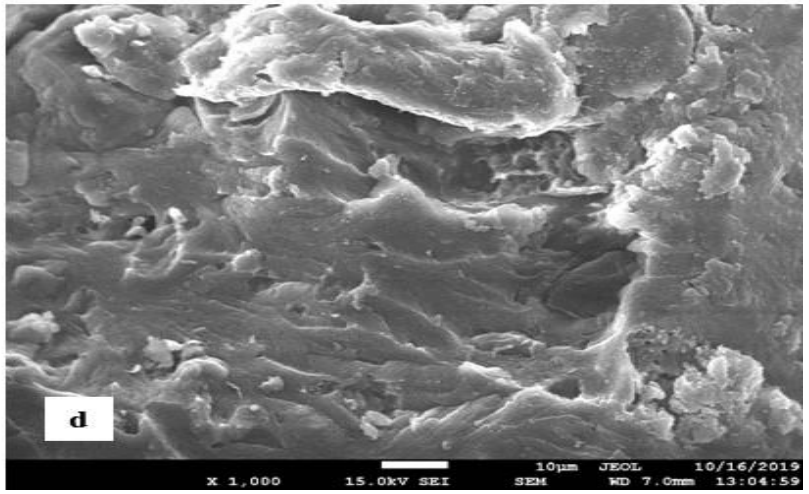
d
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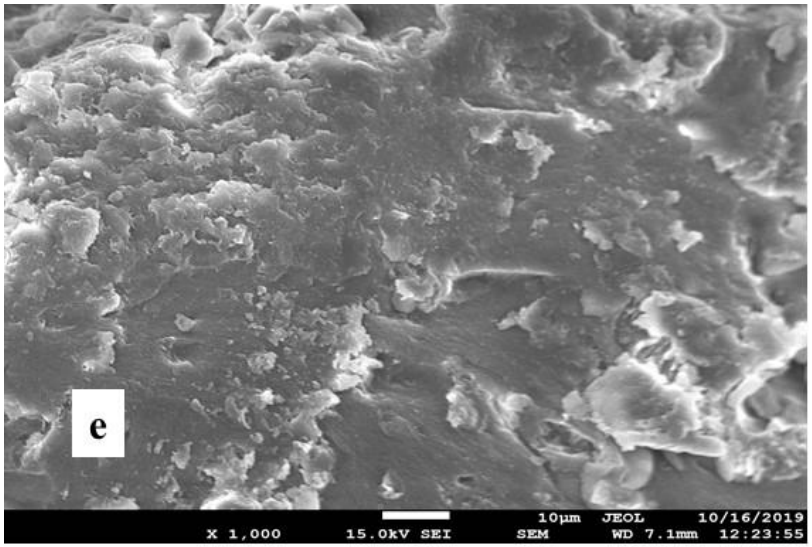
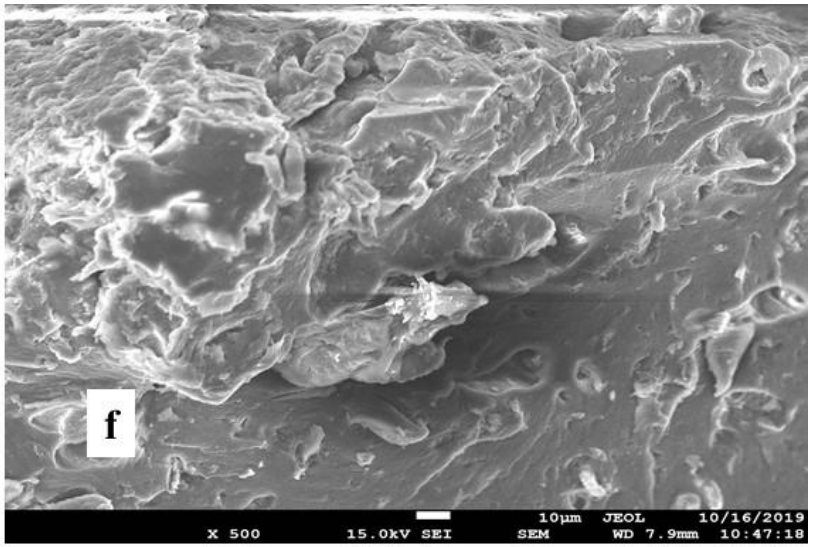
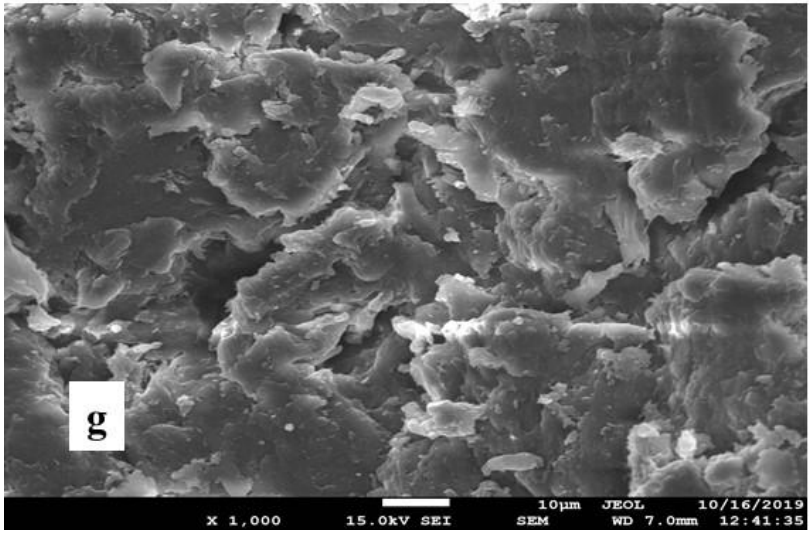
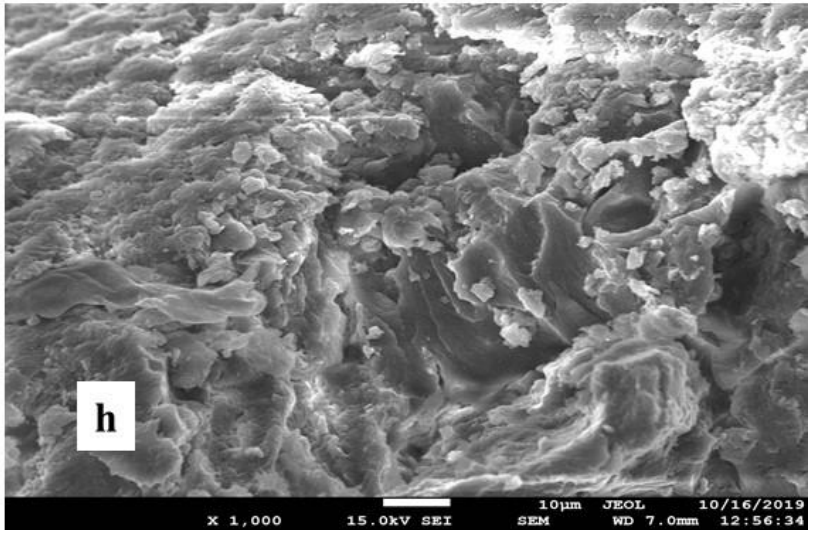

e
X 1,000 15.0kV SEI 10µm JEOL SEM WD 7.1mm 10/16/2019 12:23:55


f
X 500 15.0kV SEI 10µm JEOL SEM WD 7.9mm 10/16/2019 10:47:18


g
X 1,000 15.0kV SEI 10µm JEOL SEM WD 7.0mm 10/16/2019 12:41:35


h
X 1,000 15.0kV SEI 10µm JEOL SEM WD 7.0mm 10/16/2019 12:56:34

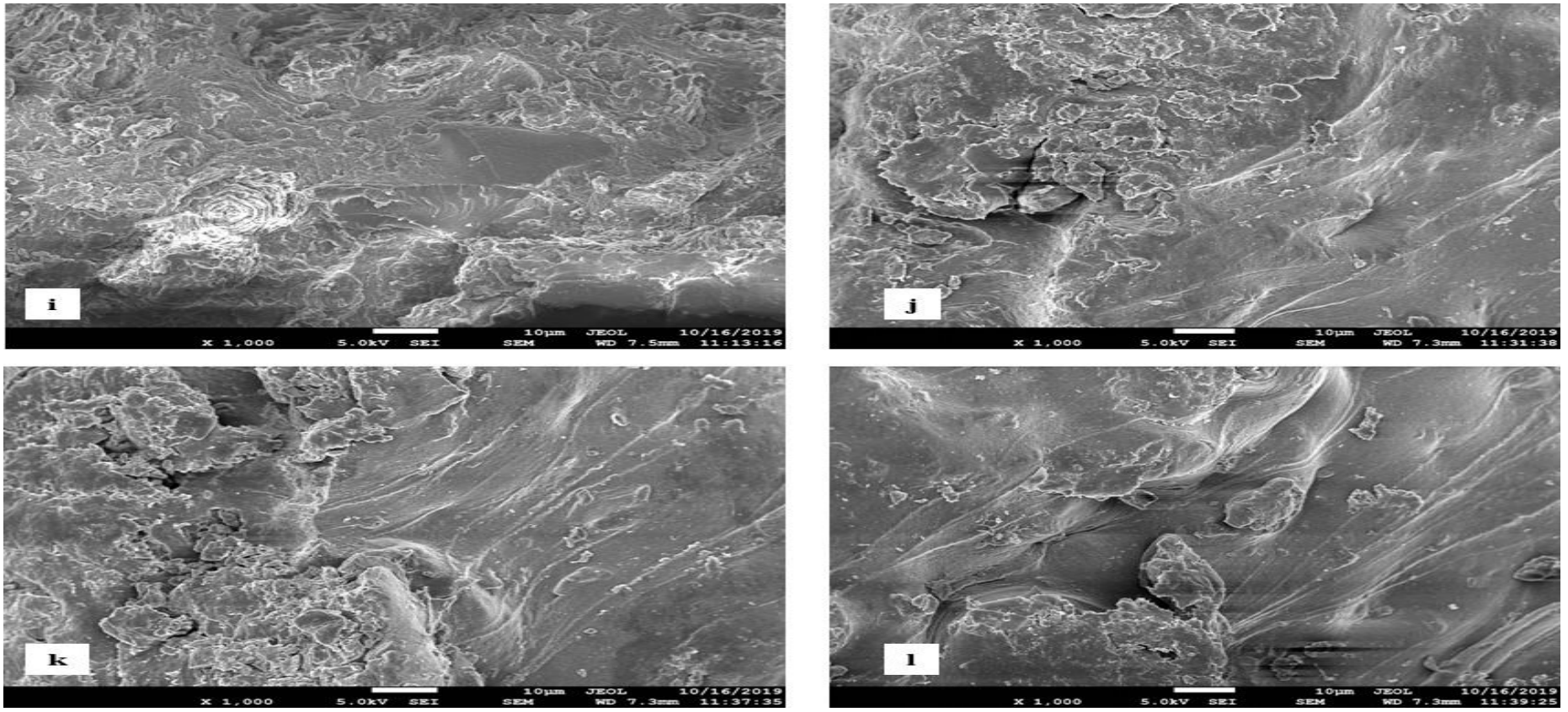


Fig. 13: SEM micrograph images of beads; AWC beads after adsorption of (a) cefixime, (b) cefotaxime, (c) gemifloxacin (d) gatifloxacin, CAW beads after adsorption of (e) cefixime, (f) cefotaxime, (g) gemifloxacin (h) gatifloxacin, WAC beads after adsorption of (i) cefixime, (j) cefotaxime, (k) gemifloxacin (l) gatifloxacin.

6.5.3. EDX analysis

The Energy dispersive spectra (EDX) define the elemental peaks of ACW, CAW and WAC beads and the peaks confirms presence of carbon, oxygen and in minor amount nitrogen also (**Fig. 14** (**a, b, c**)).

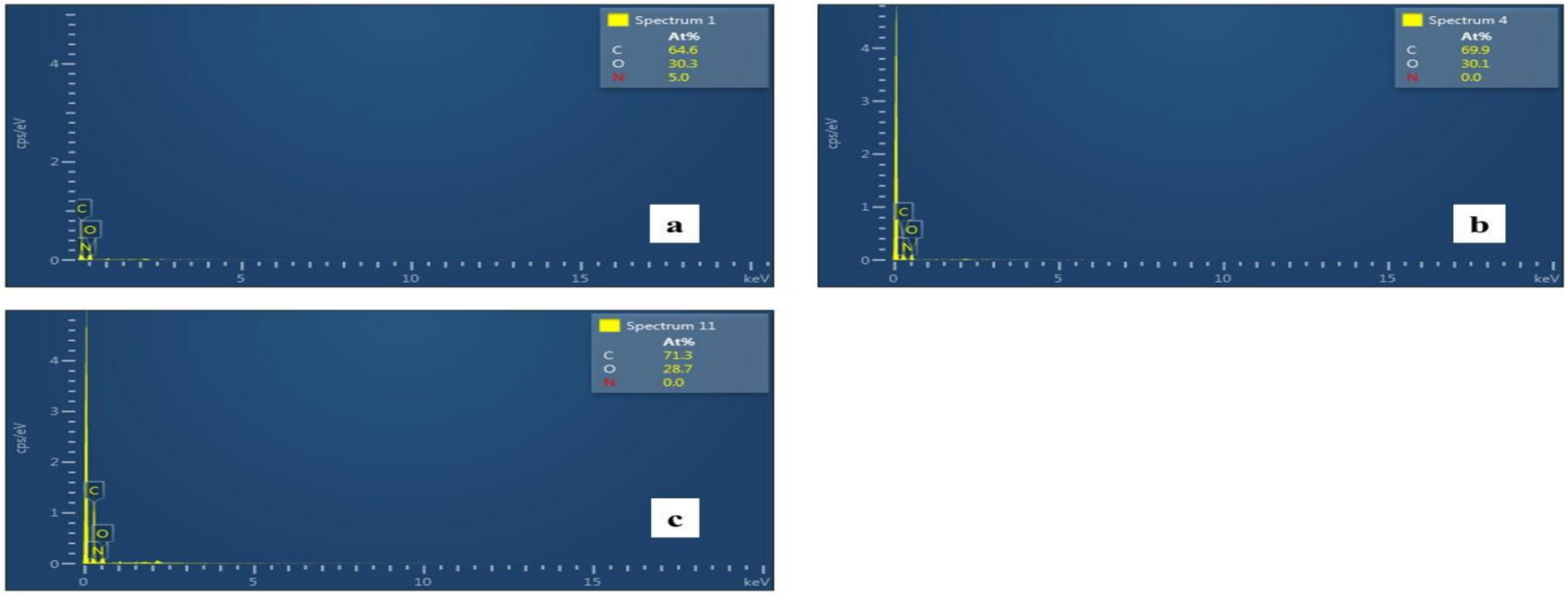


Fig. 14: EDX images of AWC beads (a), CAW beads (b) and WAC beads (c).

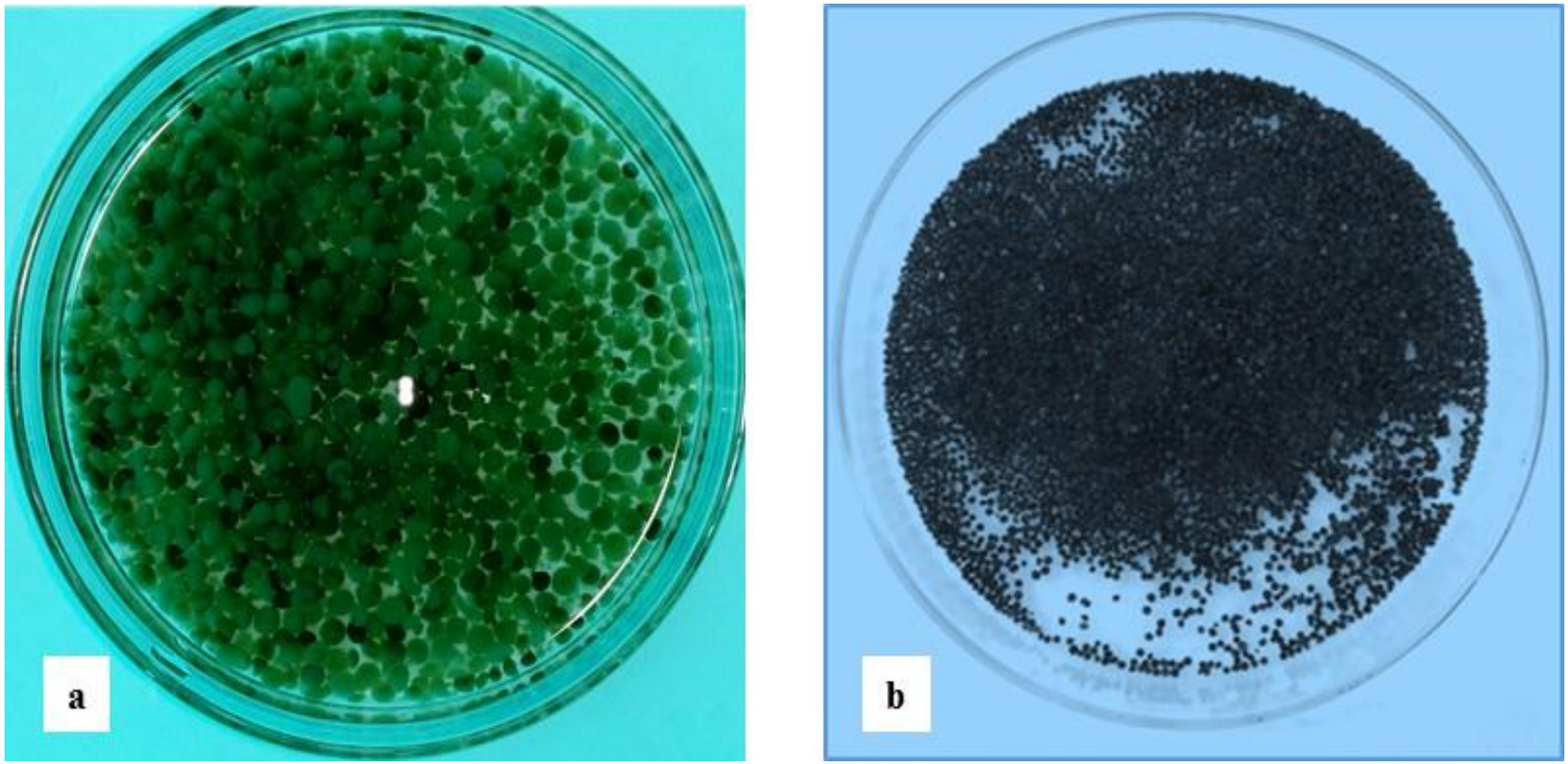


Fig. 15: Prepared (a) wet beads (b) dry beads.

6.5.4. Results and discussion of antibiotic adsorption

6.5.4.1. Adsorption of Cefixime

6.5.4.1.1. Effect of adsorbent dosage on adsorption

The adsorption dosage varied from 0.1 to 1.0 g/50ml, pH 7.0 and initial concentration of antibiotic 30 mg/L. The removal efficiency of adsorbents are shown in **Fig. 16a**. The adsorption uptake increased from 76 to 89% on AWC beads, 79 to 88% on CAW beads and 79 to 90% on WAC beads. The results obtained can be explained by the fact that by raising the dosage of adsorbents, the active sites of adsorbents surface will increase and more antibiotic molecules can be adsorbed on the surface of the beads. Similar trends in adsorption were earlier reported (Yadav *et al*., 2018; Ahsan *et al*., 2018).

6.5.4.1.2. Effect of contact time on adsorption

Different time intervals (30-180 min) were used to determine the equilibrium time. The removal of cefixime antibiotic increases with the increase in exposure time (**Fig. 16b**). The equilibrium time for CAW and AWC beads was 120 min exhibited adsorption of 77.2% of the antibiotic. On WAC beads, equilibrium was attained after 90 mins with 80.1% adsorption. The experimental data showed that the adsorption percentage for cefixime reached 71.1% within 30 min and upto 84% within 180 min. Thus exhibiting 18.31 % removal increase from the start of experimental time period. Similarly AWC and CAW beads showed 32.75% removal increase within 180 min. In the beginning, the percentage removal of cefixime was rapid due to the larger adsorbent surface area available, but it slowly decreased over time due to saturation of the active site which does not permit further adsorption (Azarpira and Balarak, 2016).

6.5.4.1.3. Effect of pH on biosorption of antibiotic

The pH was varied from 3-11 in order to investigate percentage removal of cefixime on synthesized beads. From the graphs (**Fig. 16c**) it is clear that optimum adsorption efficiency on AWC (79%) and CAW (81%) beads were observed at pH 7.0. Whereas on WAC (82%), maximum adsorption was observed at pH 5.0. The increase in pH after 7.0 cause decrease in adsorption percentage for all the three different

combinations. The pH of solution is a significant factor which affects the adsorption of cefixime antibiotic, as it can influence surface charges of the adsorbent (Yadav *et al*., 2018).

6.5.4.1.4. Effect of initial concentration on percentage adsorption

Cefixime adsorption was studied by changing the antibiotic concentration from 10 to 50 mg/L, while keeping other parameters constant. The percentage removal of cefixime antibiotic decreased when initial concentration increased from 10-50 mg/L (**Fig. 16d**). The adsorption decreased from 93 to 60% in case of AWC and WAC beads and 94 to 65% in case of CAW beads. In the beginning, the percentage removal of cefixime was rapid due to the larger surface area of adsorbent available, but it slowly decreased due to saturation of the active site which does not permit further adsorption (Azarpira and Balarak, 2016).

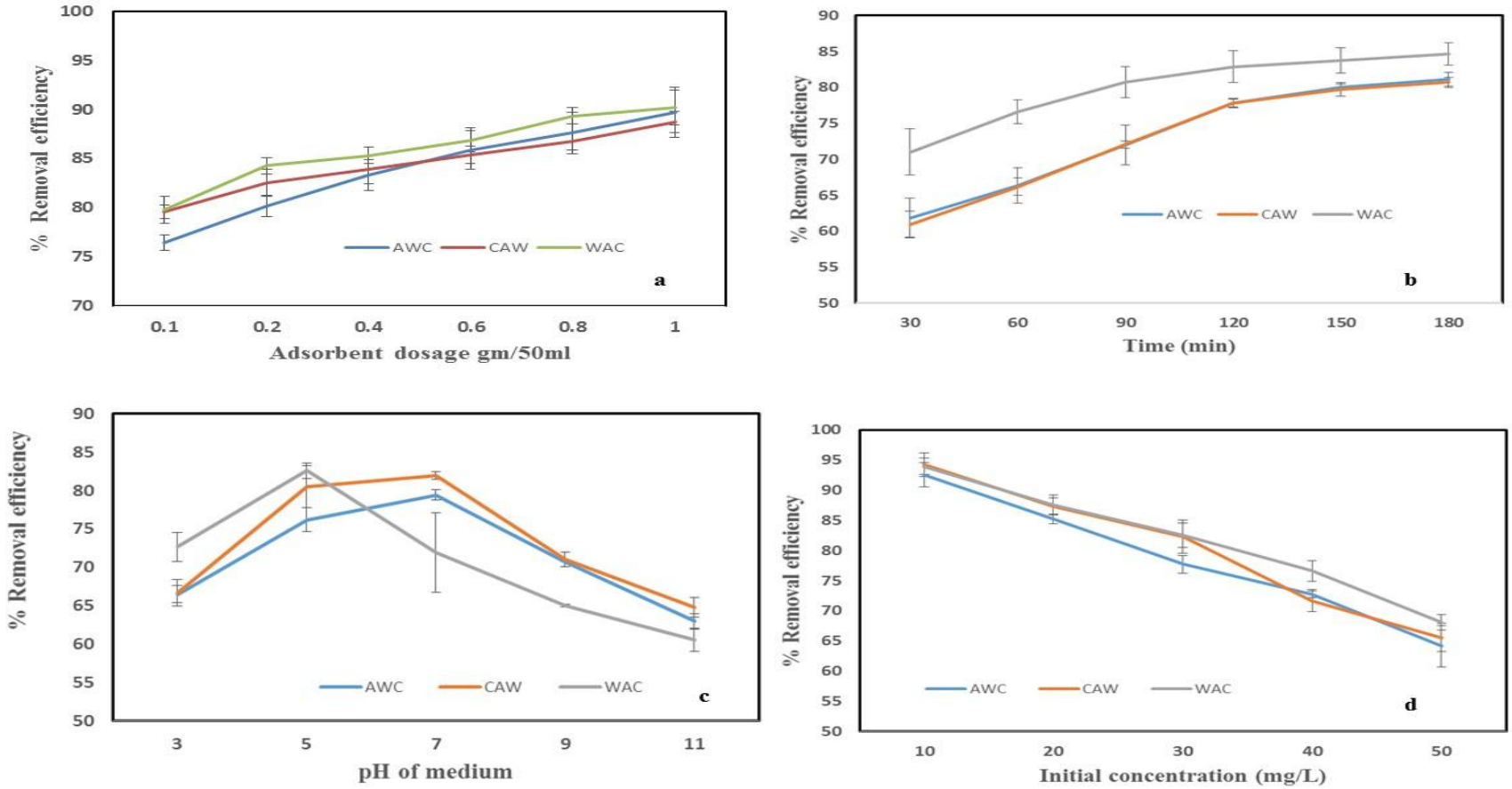


Fig. 16: (a) Adsorbent dosage effect on the removal of cefixime onto AWC, CAW and WAC beads, (b) pH effect on the removal of cefixime onto AWC, CAW and WAC beads, (c) Effect of contact time on the removal of cefixime onto AWC, CAW and WAC beads and (d) Effect of initial concentration on the removal of cefixime onto AWC, CAW and WAC beads

6.5.4.1.5. Adsorption isotherm results

The Langmuir and Freundlich isotherms were used to model the experimental data (**Fig.17a, 17b).** The isotherm constants are presented in **Table 7**. It can be seen that the experimental data fitted best with Langmuir model for AWC and CAW beads and Freundlich model for WAC beads with higher value of correlation coefficient.

Table 7: **various constants related to adsorption isotherms**

	Langmuir isotherm				**Freundlich isotherm**		
	AWC	**CAW**	**WAC**		**AWC**	**CAW**	**WAC**
Q (mg/g)	17.409	17.182	18.179	kf (mg/g)	6.173	5.531	4.946
b (L/mg)	0.354	0.464	0.438	1/n	0 .338	0.407	0 .552
R2	0.989	0.989	0.943	R2	0. 975	0.908	0.980
SD	0.0030	0.0028	0.0070	SD	0 .016	0.037	0.020

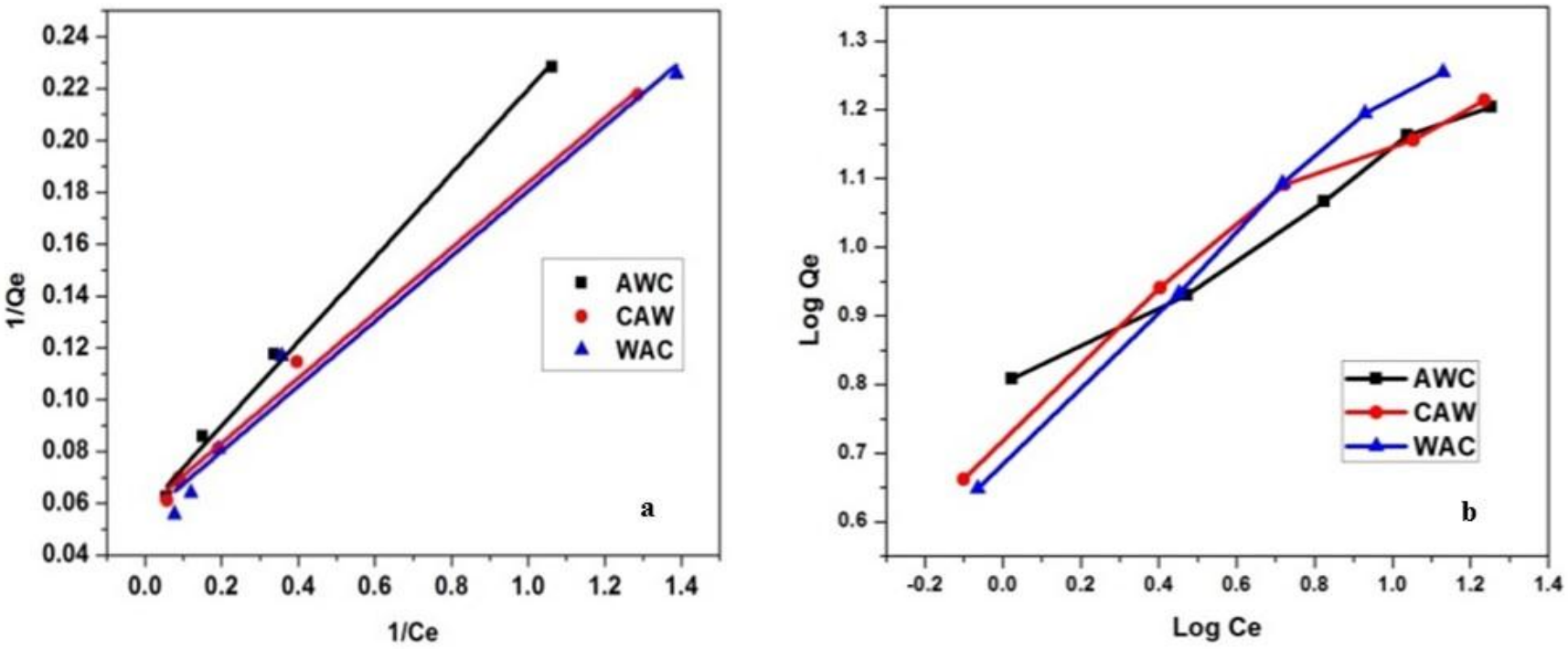


Fig. 17: Adsorption isotherms of (a) Langmuir model (b) Freundlich model.

6.5.4.1.6. Adsorption kinetics results

The adsorption kinetic models predicts adsorption mechanism and rate of adsorption onto the beads. Data was fitted to the kinetic models and results are presented in graphs (**Fig. 18a, 18b**), and associated constants are presented in **Table 8**. The straight line represents plots of log (Qe–Qt) against time (t) for pseudo ist order reaction and log (t/Qt) against time (t) for the pseudo 2nd order kinetics. It is clear from the R_2 values given in the **Table 8**. that AWC (0.993), CAW (0.994) and WAC (1) bead adsorption follow Lagergren pseudo second order better than Lagergren first order kinetic model.

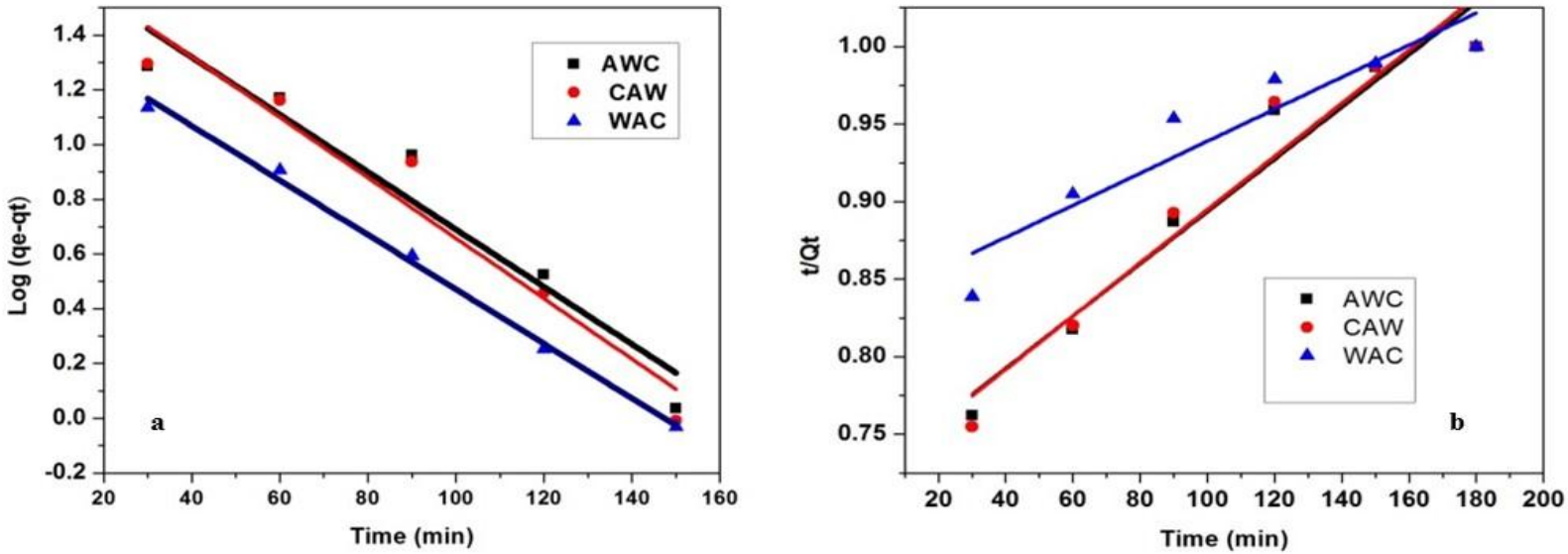


Fig. 18: (a) Pseudo first order kinetic model (b) Pseudo second order kinetic model.

Table 8: Representing the comparison of various kinetic models

	Pseudo-first-order constants				Pseudo-second-order constants		
	AWC	**CAW**	**WAC**		**AWC**	**CAW**	**WAC**
K_1 (ads) (min−1)	0.024	0.025	0.022	K_2 (g/mg/min)	0 .0006	0.0006	0.0013
Qe (mg/g)	54.93	57.93	29.39	Qe (mg/g)	88.49	88.18	88.41
-	-	-	-	h (g/mg/min)	5.19	5.18	10.69
R^2	0.832	0.858	0.990	R^2	0.993	0.994	1.0
SD	0.035	0.153	0.035	SD	0.000	0.030	0.008

6.5.4.2. Adsorption results of cefotaxime

6.5.4.2.1. Effect of adsorbent dosage on adsorption

The effect of adsorbent amount on the removal of cefotaxime from aqueous solution onto synthesized beads were investigated in the range of 0.1- 1.0g/50ml (**Fig. 19a)**. The result showed that cefotaxime adsorption increased with the increase in concentration of adsorbent and maximum adsorption was obtained at 1.0 g/50 ml. The adsorption on WAC beads was 90.5%, on AWC beads 88% and 85.7% on CAW beads respectively. The amount of adsorption increased due to an increase in active sites on the surface of beads. But with the increase in adsorption concentration there was not that much increase in antibiotic adsorption. Therefore, 0.1 g/50ml adsorbent dosage was appropriate for all subsequent experiments.

6.5.4.2.2. Effect of contact time on adsorption

The adsorption of antibiotic on the adsorbents in a medium is influenced by reaction time and the selection of suitable adsorption time have economic benefits. The influence of contact time on the uptake of cefotaxime onto beads was studied and shown in **Fig. 19.b**.The initial adsorption of antibiotic was quick within first 120 minutes and after that shows gradual increase. The maximum adsorption efficiency on AWC beads was 84.0%, on CAW beads was 86.3% and on WAC beads was 85.0% respectively. The presence of large number of vacant sites on bead surface at initial time results in quick adsorption of antibiotics. And with the increase in contact time, sorption sites on the surface of synthesized beads are close to saturation which results in reduction of antibiotic adsorption (Mahmood and Abdulmajeed, 2017).

6.5.4.2.3. Effect of pH on biosorption of antibiotic

The effect of pH solution is an important parameter which controls the adsorption process (Ahsan *et al*., 2018; Yadav *et al*., 2018). **Fig. 19c**, shows the effect of pH (3.0 to 11.0) on the adsorption of cefotaxime antibiotic. Optimum adsorption of antibiotic was observed at pH 5.0, with cefotaxime removal rate of about 83.42% on AWC, 84.58% on CAW and 84.80% on WAC beads respectively. However, with the further increase in pH, adsorption was observed to be decreasing (Kumar *et al*., 2017).

6.5.4.2.4. Effect of initial concentration on adsorption

The initial concentration of antibiotic is an important factor, which influences the extent of contaminant uptake from the solution. The different concentrations of cefotaxime i.e, 10 to 50 mg/L was chosen, keeping all other conditions constant. The removal percentage decreased from 94% to 68%, 83% to 56%, and 85% to 49% on WAC, AWC and CAW beads respectively (**Fig. 19d**). This indicates that the initial concentration strongly affects the removal efficiency. The reason being, more available binding sites were being covered as cefotaxime concentration increased (Azarpira and Balarak, 2016).

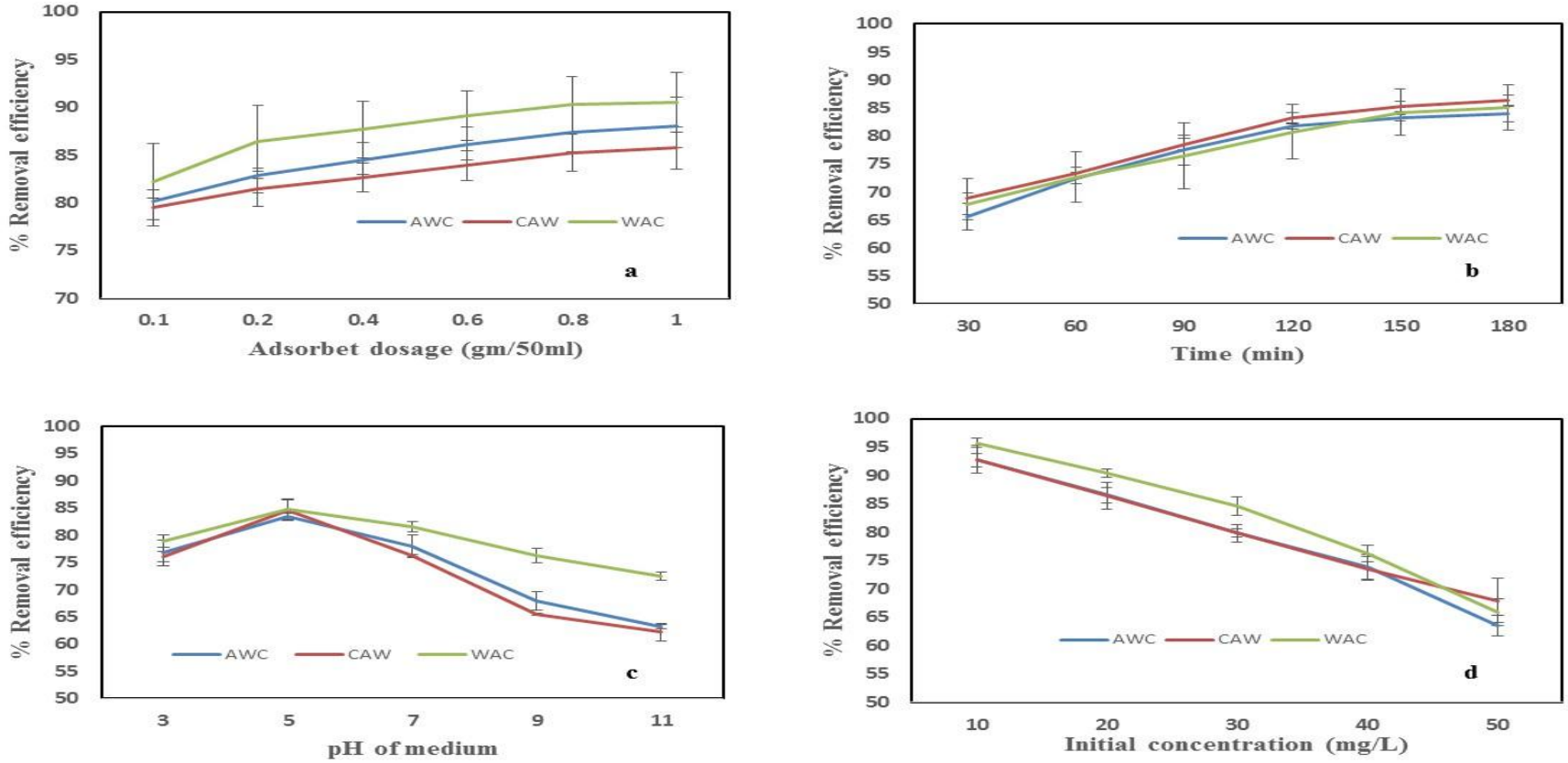


Fig. 19: (a) Adsorbent dosage effect on the removal of cefotaxime onto AWC, CAW and WAC beads, (b) pH effect on the removal of cefotaxime onto AWC, CAW and WAC beads, (c) Effect of contact time on the removal of cefotaxime onto AWC, CAW and WAC beads and (d) Effect of initial concentration on the removal of cefotaxime onto AWC, CAW and WAC beads.

6.5.4.2.5. Adsorption isotherm results

The Langmuir and Freundlich isotherms were used to model the experimental data (**Fig.20a, 20b).** The isotherm constants are presented in **Table 9**. It can be seen that the experimental data fitted best with Langmuir model for WAC beads and Freundlich model for CAW beads with higher value of correlation coefficient. Whereas, ACW beads follows both Langmuir and Freundlich isotherm models.

Table 9: Various constants related to adsorption isotherms

Langmuir Constants					**Freundlich Constants**		
	ACW	**CAW**	**WAC**		**ACW**	**CAW**	**WAC**
Q	17.813	17.775	15.540	K_f	5.694	5.623	5.855
B	0.495	0.530	1.047	1/n	0.430	0.453	0.343
R^2	0.963	0.938	0.950	R^2	0.964	0.998	0.935
SD	0.005	0.006	0.005	SD	0.023	0.005	0.024

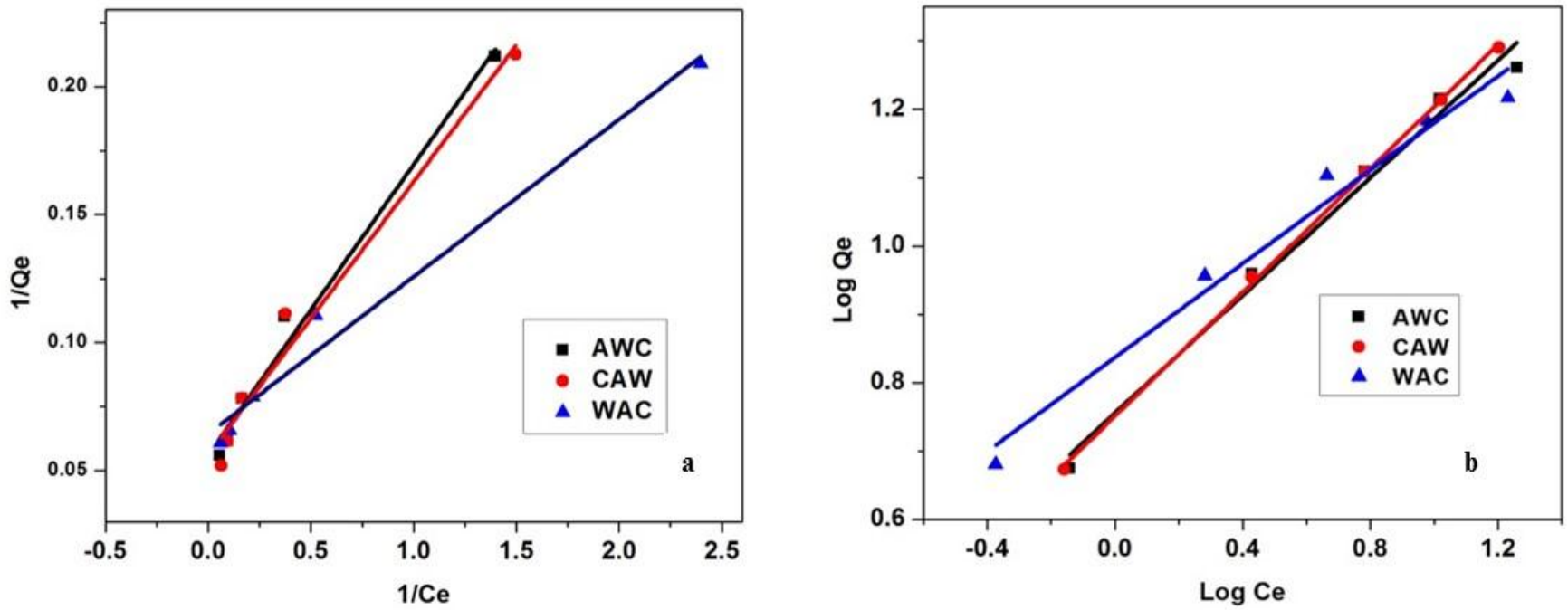


Fig. 20: (a) Langmuir Isotherm for synthesized beads and (b) Freundlich isotherm for synthesized beads

6.5.4.2.6. Adsorption kinetic results

In the **Fig. 21 (a, b)** the straight line represents plots of log (Qe–Qt) against time (t) for pseudo1st order reaction and log (t/Qt) against time (t) for the pseudo 2nd order kinetics. The parameters k_1, k_2, Qe and R^2 were calculated from these plots as given in **Table 10**. Initially, large concentration difference occurs between the aqueous and the adsorbent surface which is accountable for a quicker adherence of solute onto the adsorbent surface. However, after a certain period, slow intraparticle diffusion occurs at the internal adsorption sites of the adsorbent. On the basis of R^2 (**Table 10**) values, adsorption follows Lagergren pseudo second order kinetic model on all the types of beads.

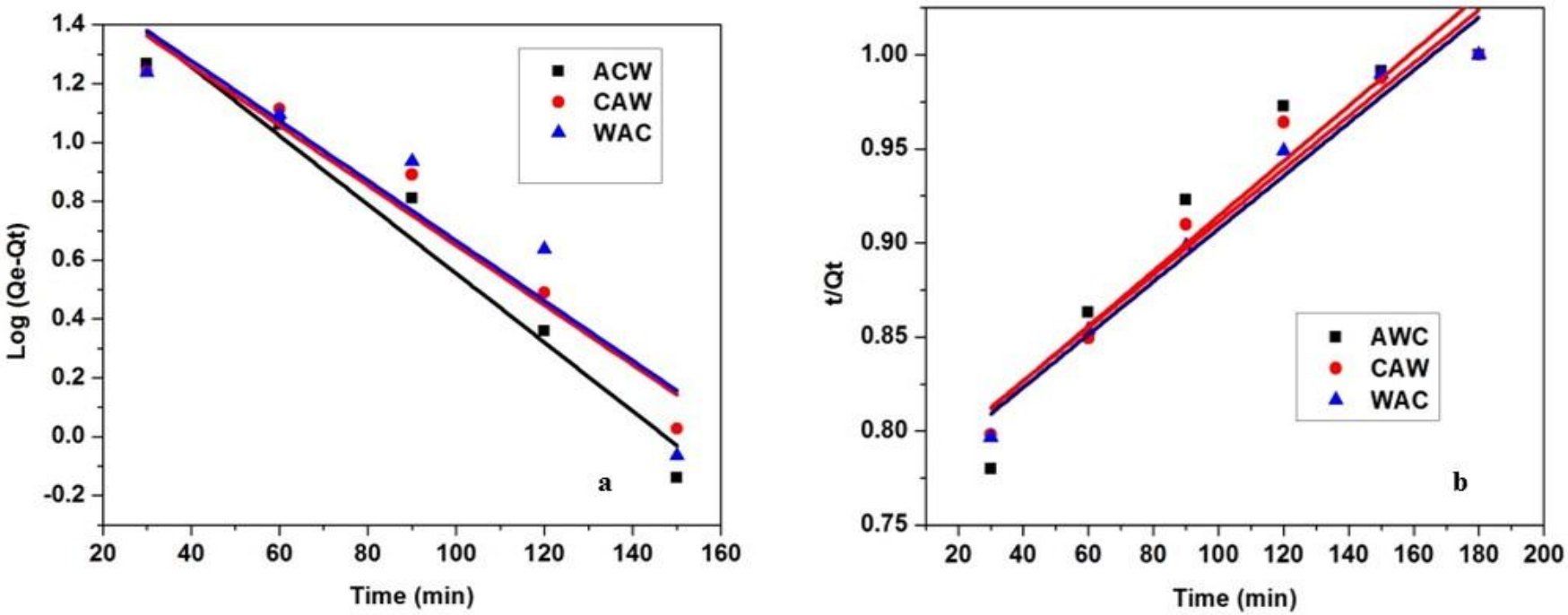


Fig. 21: (a) Lagergren Pseudo First order and (b) Lagergren Pseudo Second order

Table 10: Comparison of various kinetics models

	Pseudo-first-order-constants				Pseudo-second-order constants		
	AWC	CAW	WAC		AWC	CAW	WAC
Qe	53.210	46.550	48.640	Qe	90.90	100	100
K_1	0.027	0.023	0.023	H	6.916	6.937	6.625
R^2	0.906	0.863	0.703	k_2	0 .000	0 .000	0 .000
SD	0.129	0.130	0.210	R^2	0.998	0.996	0.994
				SD	0.015	0.023	0.029

6.5.4.3. Adsorption results of gemifloxacin

6.5.4.3.1. Effect of adsorbent dosage on percentage adsorption

With the increase in adsorbent dosage removal efficiency was also increasing. The adsorption increased from 81% to 89% on AWC beads, 81% to 90% on CAW beads and 83% to 92% on WAC beads respectively (**Fig. 22a**), when the adsorbent dosage was increased from 0.1-1.0 g/50ml and keeping all the other conditions constant. The amount of adsorption increased due to the increase in active sites on the surface of beads (Kakavandi *et al*., 2014; Ahsan *et al*., 2018). A direct relation could be established by stating the influence of available surface area and charge on the beads to interact with antibiotics (Chen *et al*., 2014). The increase in adsorbent amount results in slight increase in antibiotic adsorption. Therefore, for consequent adsorption studies, 0.1 g/50ml adsorbent dosage was appropriate for all subsequent experiments.

6.5.4.3.2. Effect of contact time on percentage adsorption

The effect of reaction time (0-180 min) on the removal of gemifloxacin antibiotic on synthesized beads are presented in **Fig. 22b**.The adsorption was observed to be increasing quickly till 120 min. The presence of large number of vacant sites on bead surface at initial time results in quick adsorption of antibiotics. And with the increase in contact time, sorption sites on the surface of synthesized beads are close to saturation which results in reduction of antibiotic adsorption (Azarpira and Balarak, 2016). The contact time for further studies were selected as 180 mins. The graph

depicts that adsorption percentage reached 86%, 90% and 85% in case of AWC, CAW and WAC beads respectively in 180 min.

6.5.4.3.3. Effect of pH on biosorption of antibiotic

Solution pH is an essential factor influencing the process of adsorption by affecting the activity of functional groups of the adsorbent and surface solution chemistry of contaminants. **Fig. 22c,** shows the effect of solution pH (3-11) on the removal of gemifloxacin by synthesized beads at contact time of 180 min. At lower pH, fluoroquinolone antibiotics carry positive charges due to protonation of amine groups and at higher pH, they behave as anion due to deprotonation of carboxylic groups, however; at neutral pH Zwitter ion exists (Yadav *et al*., 2018). Thus, the study was spread over with range from pH 3-11. The maximum gemifloxacin removal was observed at pH 5.0 with AWC beads (82.0 %), which is also found in a research where in amoxicillin adsorption increases from pH 2-5 as the carboxyl functional groups (-COOH) on the amoxicillin readily dissociate to carboxylate (COO^-) which increases electrostatic attraction between amoxicillin and the adsorbent (Putra *et al*., 2009; Moussavi *et al*., 2013). Maximum adsorption of gemifloxacin was found at pH 7.0 on CAW (84%) and WAC (80%) beads, because of its Zwitter ionic form which may be due to protonated amine groups that still able to help in adsorption (Yadav *et al*., 2018). The decrease in the adsorption was attributed to deprotonation of C=O groups on antibiotic and beads, which significantly cause repulsion between negative charges found on beads and antibiotics (Jiang *et al*., 2013). From the FTIR analysis it was found that all the three different beads contain OH, and CO groups which give negative charges due to oxygen containing groups (Yadav *et al*., 2018). The pH of the solution influence surface charges of the adsorbent and the structure of antibiotic molecules (Yadav *et al*., 2018) there by affects the adsorption process and further removal from synthetic water.

6.5.4.3.4. Effect of initial concentration on percentage adsorption

The effect of initial concentration of gemifloxacin on the adsorption efficiency of beads are shown in **Fig 22.d**. Removal of the gemifloxacin decreased from 95% to 65% (AWC beads), 93% to 68% (CAW beads) and 94% to 68% (WAC beads) respectively, when the initial concentration was increased from 10 to 50 mg/L. In the

beginning, the percentage removal of gemifloxacin was rapid due to the the presence of active sites on adsorbent, but later slowly down due to the saturation of these active site (Azarpira and Balarak, 2016).

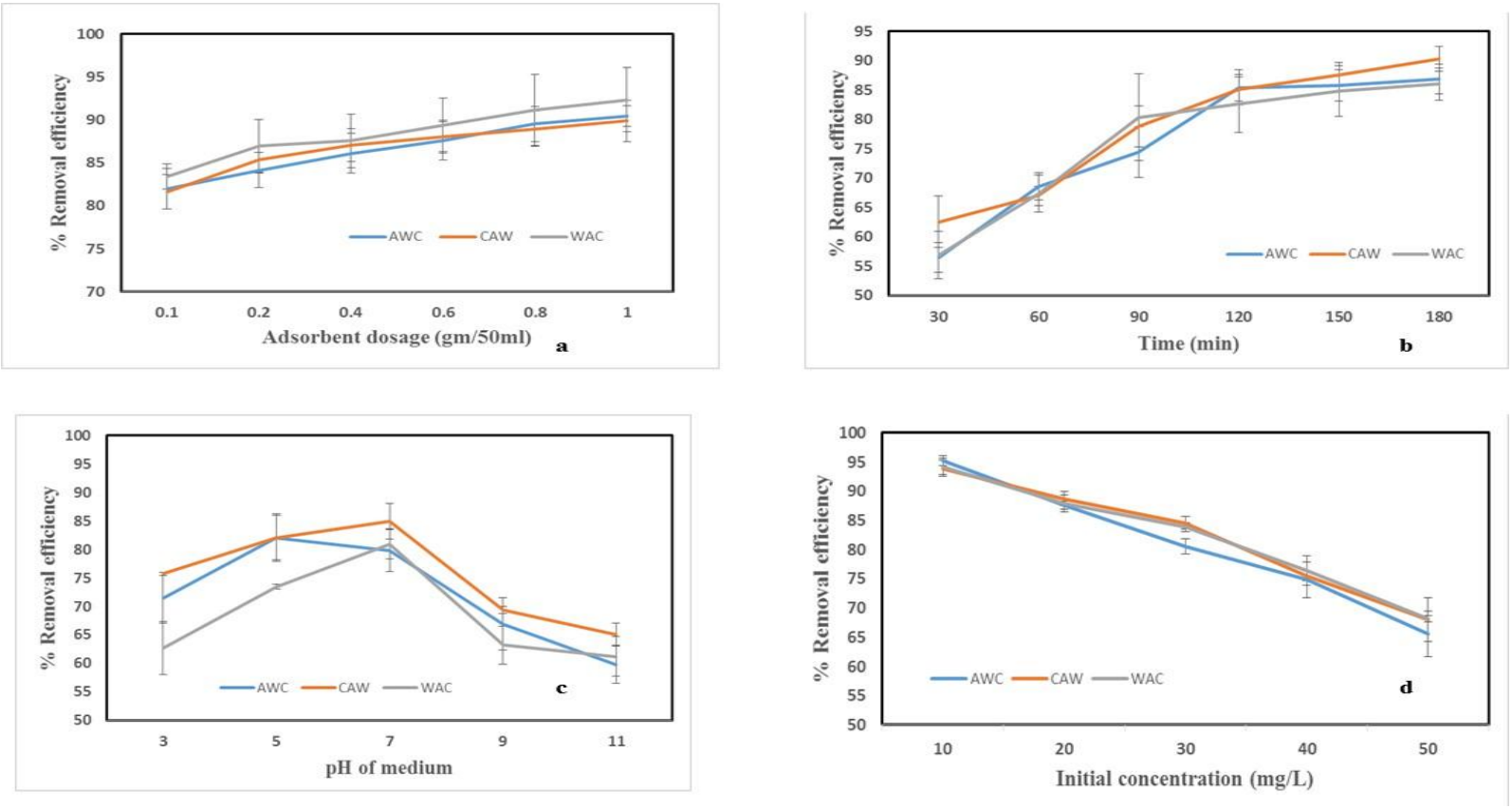


Fig. 22: (a) Adsorbent dosage effect on the removal of gemifloxacin onto AWC, CAW and WAC beads, (b) pH effect on the removal of gemifloxacin onto AWC, CAW and WAC beads, (c) Effect of contact time on the removal of gemifloxacin onto AWC, CAW and WAC beads and (d) Effect of initial concentration on the removal of gemifloxacin onto AWC, CAW and WAC beads.

6.5.4.3.5. Adsorption isotherm results

The Langmuir and Freundlich isotherms were used to model the experimental data (**Fig. 23a, 23b)**. The isotherm constants are shown in **Table 11**. The comparison of all the values of R^2 (non-linear regression coefficient) for the isotherms concludes that the Langmuir model is better followed in case of CAW beads and Freundlich adsorption better in case of AWC and WAC beads.

Table 11: Various constants related to adsorption isotherms

Langmuir Constants				**Freundlich Constants**			
	AWC	**CAW**	**WAC**		**AWC**	**CAW**	**WAC**
Q	12.47	16.887	19.877	K_f	6.290	6.147	5.194
B	1.270	0.640	0.392	1/n	0.337	0.397	0 .505
R^2	0.639	0.967	0.955	R^2	0.972	0.934	0.970
SD	0.015	0.004	0.006	SD	0.017	0.029	0.023

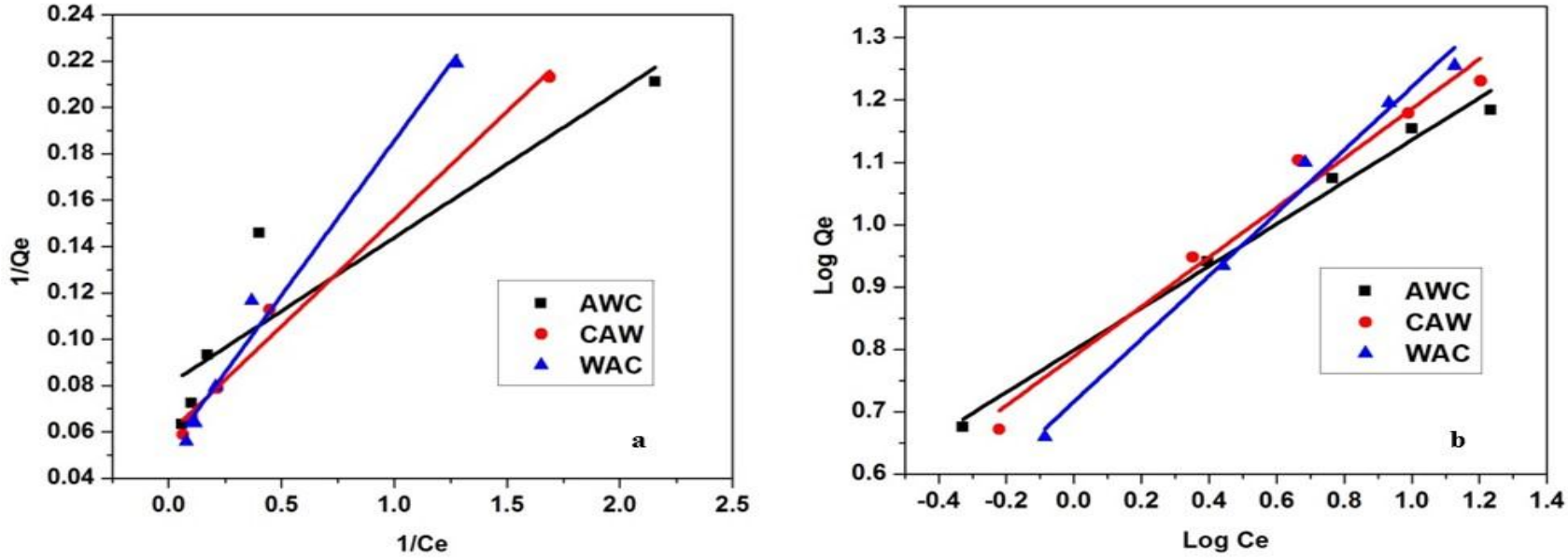


Fig. 23: (a) Langmuir Isotherm for synthesized beads and (b) Freundlich isotherm for synthesized beads.

6.5.4.3.6. Adsorption kinetics results

The straight line plots of log (Qe–Qt) against time (t) for pseudo1st order reaction and t/Qt against time (t) for the pseudo 2nd order kinetics are shown in **Fig. 24 (a-b)**. The rate parameter k_1, k_2, Qe and R_2 of gemifloxacin were computed from these plots given in **Table 12**. It is apparent on the basis of R_2 values adsorption Lagergren pseudo second order kinetic model.

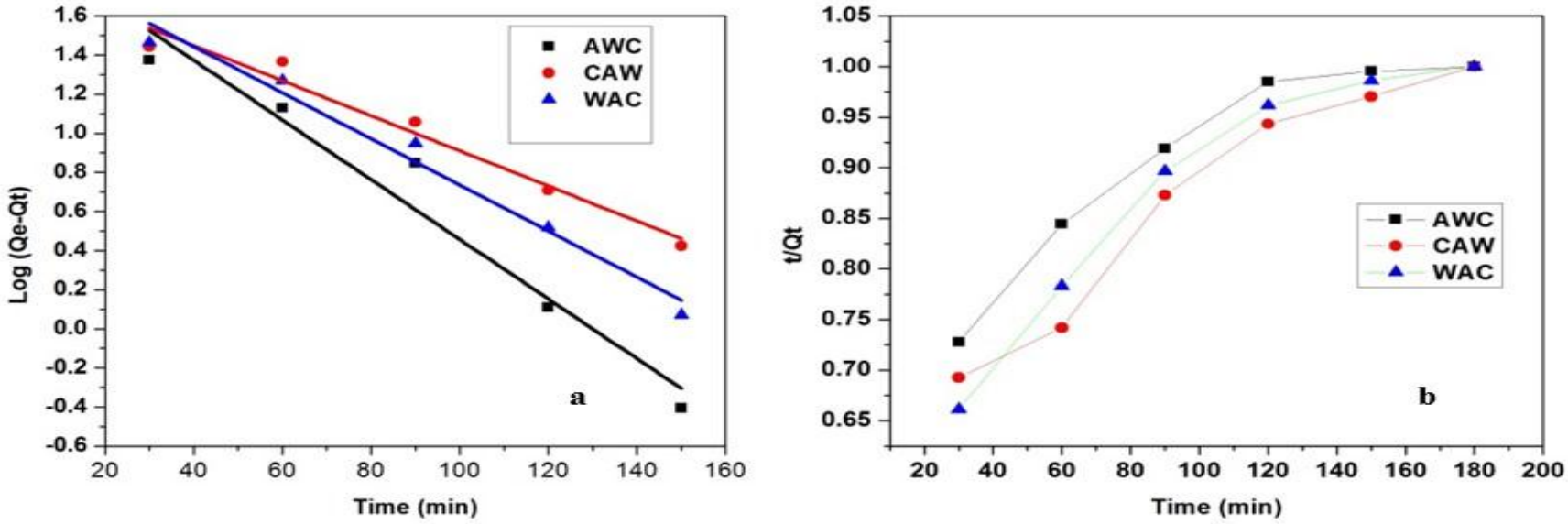


Fig. 24: (a) Lagergren Pseudo First order and (b) Lagergren Pseudo Second order

Table 12: Comparison of various kinetics models

	Lagergren Pseudo First order				Lagergren Pseudo second order		
	AWC	**CAW**	**WAC**		**AWC**	**CAW**	**WAC**
Qe	96.640	64.387	82.489	Qe	96.153	102.04	98.030
K_1	0.035	0 .020	0.027	h	5.878	4.108	4.130
R^2	0.888	0.916	0.942	k_2	0 .000	0 .000	0 .000
SD	0.187	0.094	0.102	R^2	0.998	0.986	0.997
-				SD	0.018	0.042	0.022

6.5.4.4. Adsorption results of gatifloxacin

6.5.4.4.1. Effect of adsorbent dosage on percentage adsorption

The antibiotic removal percentage increase with the increase in adsorbent dosage as shown in **Fig. 25a**. Adsorption increased from 81% to 88% on AWC beads, 80% to 88% on CAW beads and 81% to 88% on WAC beads, with the increase in adsorbent dosage from 0.1-1.0 g/50ml. The amount of adsorption increased due to an increase in surface area and active sites on the surface of beads (Kakavandi *et al*., 2014; Ahsan *et al*., 2018). A direct relation could be established by stating the influence of available surface area and charge on the beads to interact with antibiotics (Chen *et al*., 2014). But with the increase in adsorbent amount the gatifloxacin adsorption percentage increase slightly due to the decrease in adsorbent sites. Therefore, for all subsequent studies, 0.1 g/50ml adsorbent dosage was adequate as only small increase would have been accomplished using larger amount of adsorbent.

6.5.4.4.2. Effect of contact time on percentage adsorption

Different time intervals (30-180 min) were used to determine the equilibration time. The removal of gatifloxacin antibiotic increases initially and becomes persistent after the optimal exposure time (**Fig. 25b**). The percentage removal of gatifloxacin on AWC, CAW and WAC beads were 84%, 88% and 88% respectively in 180 min. The large number of active binding sites on the adsorbent surface are responsible for high adsorption rate at the initial stage, which are later occupied by the antibiotic molecules (Azarpira and Balarak, 2016).

6.5.4.4.3. Effect of pH on biosorption of antibiotic

The adsorption efficiencies of beads were studied at different pH (3-11) values with fixed initial concentration of antibiotic (30 mg/L) and time period of 180 min. At lower pH, fluoroquinolone antibiotics carry positive charges due to protonation of amine groups and at higher pH, they behave as anion due to deprotonation of carboxylic groups, however; at neutral pH Zwitter ion exists (Yadav *et al*., 2018). Thus, the study was spread over with range from pH 3-11 (**Fig. 25c**). Maximum adsorption of gatifloxacin was observed at pH 5.0 on CAW (85%), which is also found in a research where in amoxicillin adsorption increases from pH 2-5 as the carboxyl functional groups (-COOH) on the amoxicillin readily dissociate to carboxylate ($-COO^-$) which increases electrostatic attraction between amoxicillin and the adsorbent (Putra *et al*., 2009; Moussavi *et al*., 2013). The maximum gatifloxacin removal was observed at pH 7.0 with AWC beads (84%) and WAC (82%) beads , because of its Zwitter ionic form which may be due to protonated amine groups, which helps in adsorption (Yadav *et al*., 2018). Deprotonation of C=O groups were responsible for decrease in adsorption on antibiotic and beads, which significantly cause repulsion between negative charges found on beads and antibiotics (Jiang *et al*., 2013). From the FTIR analysis it was found that all the three different beads contain OH, NH and CO groups which give negative charges due to oxygen containing groups (Yadav *et al*., 2018).

6.5.4.4.4. Effect of initial concentration on percentage adsorption

Fig. 25d, shows the effect of initial gatifloxacin antibiotic concentrations on the removal efficiency of antibiotics. With the increase in antibiotic concentrations, the removal efficiency of antibiotics decrease drastically. The adsorption on AWC beads decrease from 96% to 63%, from 96% to 65% on CAW beads and 94% to 62% on WAC beads respectively. The decrease in adsorption results due to the decrease in the number of available adsorption sites. This can be explained by fact that there is reduce in active sites with increase in the initial antibiotic concentration (Azarpira and Balarak, 2016).

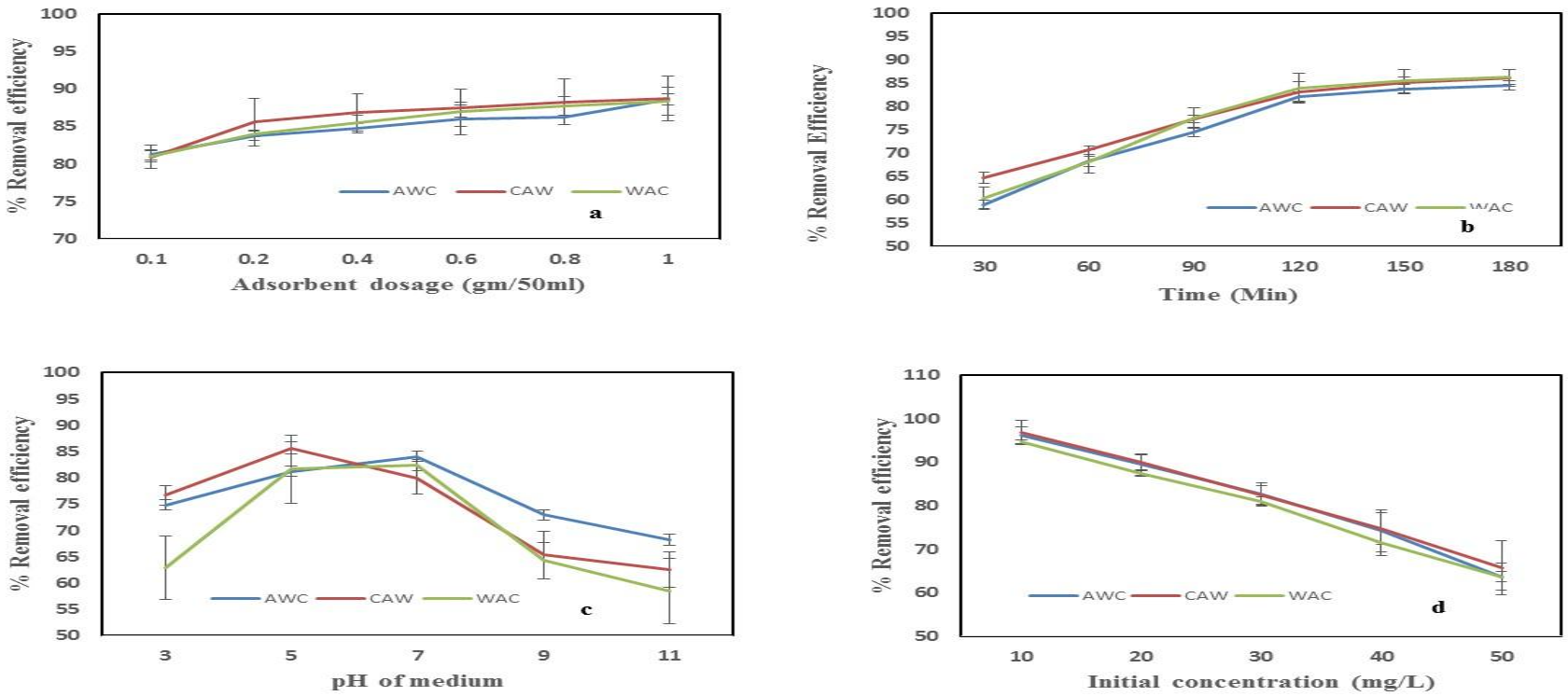


Fig. 25: (a) **Adsorbent dosage effect on the removal of gatifloxacin onto AWC, CAW and WAC beads, (b) pH effect on the removal of gatifloxacin onto AWC, CAW and WAC beads, (c) Effect of contact time on the removal of gatifloxacin onto AWC, CAW and WAC beads and (d) Effect of initial concentration on the removal of gatifloxacin onto AWC, CAW and WAC beads.**

6.5.4.4.5. Adsorption isotherm results

The Langmuir and Freundlich isotherms were used to model the experimental data (**Fig. 26a, 26b**).The determined isotherms isotherm constants are shown in **Table 13**. The comparison of all the values of R^2 (non-linear regression coefficient) for the isotherms concludes that the Langmuir model is better followed in case of ACW beads and Freundlich adsorption better in case of CAW and WAC beads.

Table 13: Various constants related to adsorption isotherms

	Langmuir Constants				Freundlich Constants		
	ACW	CAW	WAC		ACW	CAW	WAC
Q	14.531	13.637	8.270	K_f	6.836	7.359	3.405
b	1.274	2.557	0.895	1/n	0.321	0.297	0.338
R^2	0.992	0.851	0.926	R^2	0.957	0.992	0.963
SD	0.006	0.013	0.013	SD	0.020	0.008	0.020

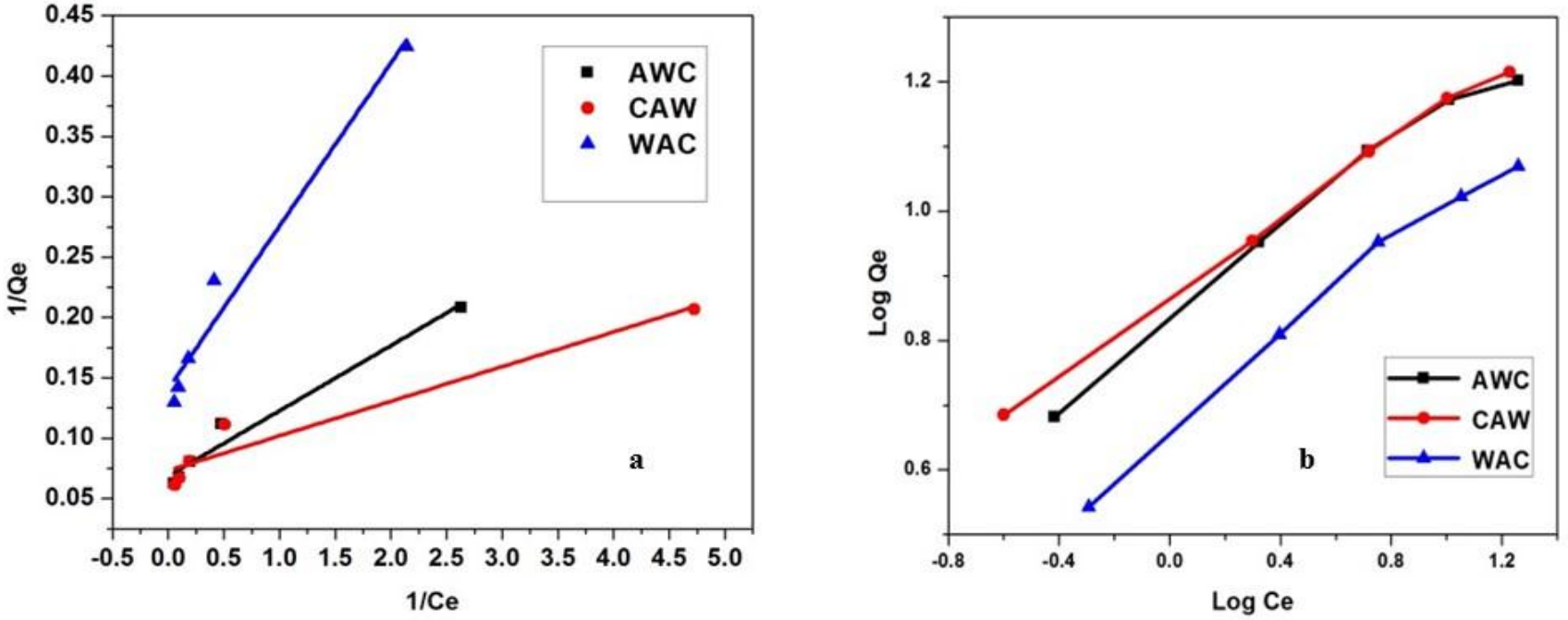


Fig. 26: (a) Langmuir Isotherm for synthesized beads and (b) Freundlich isotherm for synthesized beads.

6.5.4.4.6. Adsorption kinetic results

The straight line plots of log (Qe–Qt) against time (t) for pseudo1st order reaction and t/Qt against time (t) for the pseudo 2nd order kinetics are shown in **Fig. 27 (a-b)**. The rate parameter k_1, k_2, Qe and R_2 of gatifloxacin were computed from these plots given in **Table 14**. It is apparent on the basis of R_2 values adsorption Lagergren pseudo second order kinetic model.

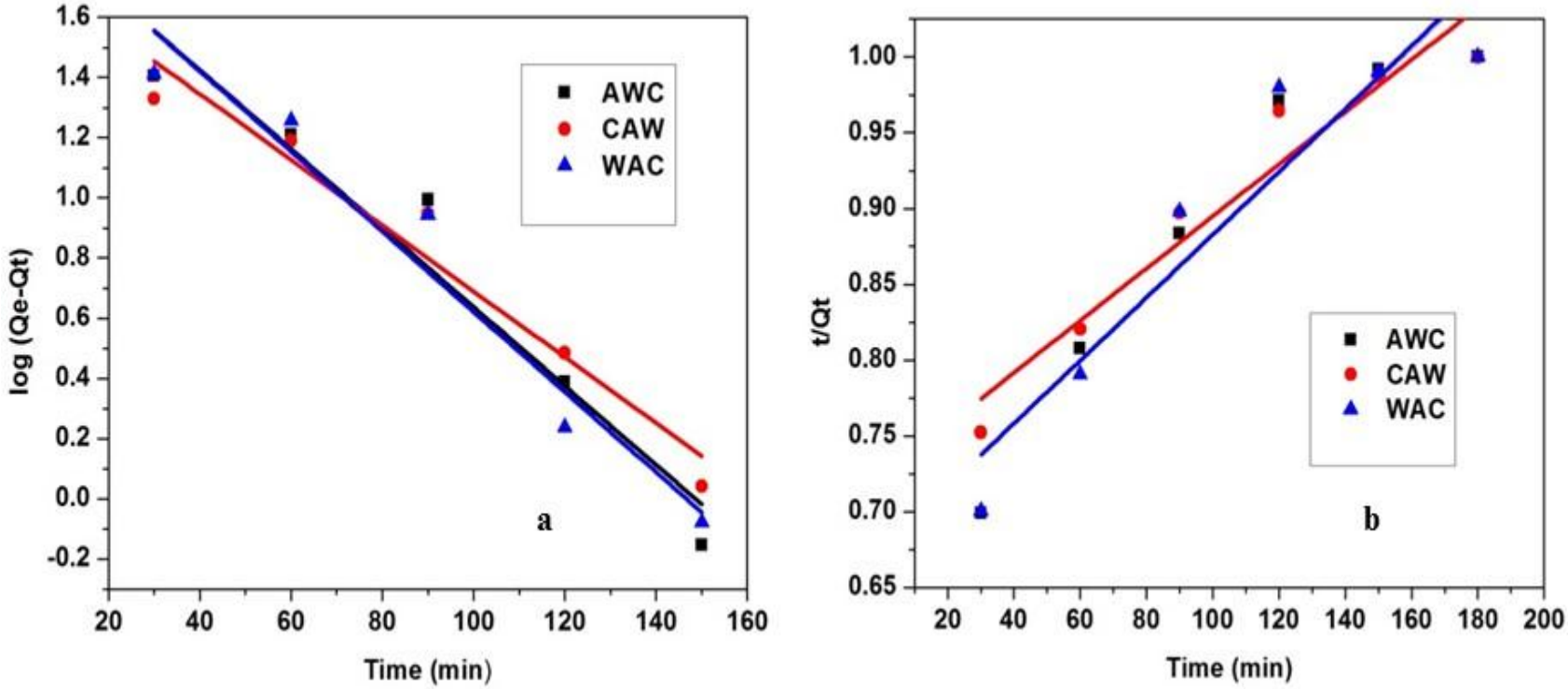


Fig. 27: (a) Lagergren Pseudo First order and (b) Lagergren Pseudo Second order

Table 14: **Comparison of various kinetics models**

	Lagergren Pseudo First order				**Lagergren Pseudo second order**		
	AWC	**CAW**	**WAC**		**AWC**	**CAW**	**WAC**
Qe	89.31	60.70	90.19	Qe	94.600	94.161	100
K_1	0.030	0.031	0 .031	H	4.567	5.559	4.623
R^2	0.854	0.880	0.875	k_2	0 .003	0.000	0 .000
SD	0.185	0.138	0.173	R^2	0 .994	0 .994	0 .992
-				SD	0.021	0 .027	0 .031

The findings revealed that with the increase in adsorbent dosage of beads from 0.1-1.0 g (**Fig. 16a, 19a, 22a, 25a**), the adsorption of cefixime, cefotaxime, gemifloxacin and gatifloxacin antibiotics were observed to be increasing. The adsorption of cefixime on AWC beads increased 17.10%, on CAW beads 11.39% and on WAC beads 13.92%. Similarly the adsorption of cefotaxime on AWC beads increased 9.88%, on CAW beads 7.88% and on WAC beads 10.23%. The adsorption of gemifloxacin on AWC beads increased 9.87%, on CAW beads 11.11% and on WAC beads 10.84%. The adsorption of gatifloxacin on AWC beads increased 8.64%, on CAW beads 10% and on WAC beads 8.64%. Similar results were observed in the previous studies as they reported increase in adsorption with the increase in adsorbent dosage Danalioglu *et al*. (2018) reported increase in adsorbent dosage of chitosan from 1.0-20 mg, increased adsorption percentage of ciprofloxacin from 17-54%. With the increase in adsorbent hydroxyapatite dosage from 0.1-5.0 g/L, the adsorption of oxytetracycline was observed to be increasing from 21-85% (Harja *et al*., 2018). Previous study reported increase in modified chitosan dosage from 5-60 mg, increased levofloxacin adsorption from 85.3-92.4% and increase in dosage from 10-200 mg increased adsorption of ceftriaxone from 7-74.2% (Mahmoud *et al*., 2020). The removal percentage of tetracycline increased from 52-93% with the increase in adsorbent from 25-100 mg. Liang *et al*. (2018) reported increase in adsorbent dosage of nanoparticles from 0.02-0.12g/L increased adsorption of antibiotics. Cephalexin adsorption was reported to increase from 33.72-44.36% onto TiO_2/GLM and GLM adsorbents with the increase in dosage amount from 0.4-4.0 g/L (Khosravi *et al*., 2018). Chitosan adsorbent concentration increment from 1-5 g, increased adsorption of tetracycline and

chlortetracycline from 95.2 and 96.3% respectively (Ma *et al*., 2019). With the increase in adsorbent dosage from 200-1000 mg the increase in carbamazepine adsorption was reported to increase from 53-87% (Naghdi *et al*., 2019). Nadoh *et al*. (2018) reported similar trend in the adsorption of antibiotics. Guava leaves increased adsorption of ciprofloxacin when adsorbent concentration increased from 20-40 mg/L (Tay and Ong, 2019). Wu *et al*. (2018) reported increase in ciprofloxacin and norfloxacin adsorption efficiency with the increase in adsorbent dosage from 1-5 mg. Ahsan *et al*. (2018) reported increased tetracycline and sulfamethoxazole adsorption onto SD-SO_3H with the increase in adsorbent dosage. The adsorption of chloramphenicol, D-cycloserine and furazolidone increased with the increase in corn stover dosage (Cheng *et al*., 2019). In a study Wu *et al*. (2019) also reported increase in fluoroquinolone and tetracycline antibiotics adsorption from 77.78-91.14%, with the increase in adsorbent dosage of biochar from 0.1-1.0 g/L. The adsorption of antibiotics can be explained by the fact that by raising the dosage of adsorbents to the correct quantity, the active sites of the adsorbent surface and available pore volume will increase and more antibiotic molecules can be adsorbed on the surface of the beads (Ahsan *et al*., 2018).

The adsorption of antibiotics were observed to be time dependent and equilibrium time for antibiotic adsorption was observed between 90-150 minutes (**Fig. 16ab, 19b, 22b, 25b**), which were also reported by previous studies (Moussavi *et al*., 2013; Fan *et al*., 2016; Mahmood and Abdulmajeed, 2017; Duan *et al*., 2019). Zhu *et al*. (2018) reported increased time period increase adsorption and becomes relatively slow subsequently. Wang *et al*. (2019) reported 120 min as equilibrium time for ciprofloxacin and enrofloxacin antibiotics adsorption on chitosan hydrogels. Tunc *et al*. (2020) also reported 120 min as equilibrium time for chlortetracycline adsorption. Adsorption equilibrium for ciprofloxacin on guava leaves was obtained at 120 minutes (Tay and Ong, 2019). Tang *et al*. (2018) observed equilibrium time of 120 min for tetracycline and chlortetracycline adsorption. Desouse *et al*. (2018) reported adsorption of azithromycin, sulfamethoxazole and ofloxacin on powdered zeolites increased with the increase in time period and equilibrium was obtained within 20 minutes and later adsorption becomes static. The optimum contact time for adsorption of tetracycline was 100 min and for doxycycline was 200 min (Rostamian *et al*.,

2018). Afzal *et al*. (2018) reported 48 hrs as equilibrium time for ciprofloxacin adsorption on chitosan/biochar hydrogel beads. The equilibrium time reported were 110 min for adsorption on Cu-X zeolite (Rahimi *et al*., 2018). Nadoh *et al*. (2018) reported equilibrium time of 30 min and further adsorption remains constant. The equilibrium time reported was 120 minutes for tetracycline and chlortetracycline on chitosan (Ma *et al*., 2019). With the increase in contact time the adsorption efficiency was observed to be increasing and equilibrium was obtained after 6 hrs on nanoparticles (Liang *et al*., 2018). Khosravi *et al*. (2018) reported increase in adsorption of cephalexin with the increase in time period and equilibrium was attained after 60 minutes. Khanday *et al*. (2019) reported equilibrium time of 4 hrs for adsorption of cephalexin onto chitin. Dao *et al*. (2018) reported 90 min equilibrium time for cefixime adsorption. Danalioglu *et al*. (2018) reported 60 min equilibrium time for ciprofloxacin adsorption on chitosan. In the beginning, the percentage removal of antibiotics were rapid due to the presence of large surface area on adsorbents, but it slowly decreased over time until equilibrium is attained. It is due to the saturation of active sites on adsorbents which does not permit further adsorption of antibiotics (Azarpira and Balarak, 2016).

The maximum adsorption of antibiotics in our studies was observed between pH 5.0-7.0 (**Fig. 16c, 19c, 22c, 25c**). Many other researchers reported similar trend onto other adsorbents used during their study. Feng *et al*. (2018) reported optimum pH range of 5.0-7.0 for the adsorption of norfloxacin (91%). Fu *et al*. (2019) reported maximum adsorption capacity of ciprofloxacin on modified waste grapefruit peel at pH 7.0. Liang *et al*. (2018) also reported adsorption increased with the increase in pH from 3.0-7.0, and later decreasing trend in adsorption was observed. Tunc *et al*. (2020) reported increased adsorption of chlortetracycline with the increase in pH from 3.0-7.0. Mahmoud *et al*. (2020) reported maximum adsorption of levofloxacin on NBent-$NTiO_2$ nanocomposite at pH 5.0. Chen *et al*. (2019) reported maximum adsorption of sulfamethazine at pH 6.0 onto biochar. Chahm *et al*. (2019) reported pH 8.0 as optimum for the adsorption of norfloxacin antibiotic onto activated termite feces. Afzal *et al*. (2018) reported constant adsorption of ciprofloxacin adsorption onto chitosan/biochar hydrogel beads between pH 3-10. Ahsan *et al*. (2018) reported 7.0 pH as optimum for tetracycline adsorption on SD-SO_3H. Naghdi *et al*. (2019) reported

the optimum pH of 6.0 for carbamazepine adsorption. The pH 5-8 was found to be optimum for the adsorption of chloramphenicol, D-cycloserine and furazolidone onto corn stover adsorbent (Cheng *et al*., 2019). Soares *et al*. (2019) reported maximum adsorption of sulfamethoxazole on TMC based-magnetic particles at pH 5.0 better for adsorption experiments. The optimum pH for azithromycin and ofloxacin adsorption was 6.0 and 8.0 respectively (Desouse *et al*., 2018). Tetracycline adsorption was observed to be maximum at pH 5.0-7.0 (Tang *et al*., 2018). Zhu *et al*. (2018) reported maximum adsorption of ofloxacin at pH 7.0. Adsorption of ciprofloxacin and norfloxacin antibiotics were also reported to be increasing with the increase in pH from 4.0-7.0 and later decreasing (Wu *et al*., 2018). Similar results were reported by Mahmoud *et al*. (2020), they found 93.3% adsorption at pH 5.0 for ceftriaxone. Metronidazole and sulfamethoxazole adsorption on walnut shell was observed maximum at pH 6.0 (Teixeira *et al*., 2019). The optimum pH of 6.0 was observed for the adsorption of ciprofloxacin on nancomposite (Nadoh *et al*., 2018). Rahimi *et al*. (2019) reported pH 5.0 as optimum for the adsorption of tetracycline on Cu-X zeolite. Rostamian *et al*. (2018) reported pH 6.0 and 7.0 as optimum for tetracycline and doxycycline adsorption on graphene oxide nanosheets. The maximum adsorption of ciprofloxacin on mesoprous silica was observed between pH 4.0-8.0 (Sousa *et al*., 2018). Dai *et al*. (2020) also reported highest adsorption capacity of tetracycline on biochar at pH 7.0. The maximum adsorption of tetracycline and chlortetracycline on chitosan was observed between pH 5.0-7.0 (Ma *et al*., 2019). With the increase in pH from 3.0-7.0 adsorption of ciprofloxacin was observed to be increasing and later decreasing onto nanoparticles (Liang *et al*., 2018). Adsorption of cephalexin onto chitin was observed to be increasing from 58-67% with the increase in pH from 3.0-7.0 and later was decreasing (Khanday *et al*., 2019). Ifebajo *et al*. (2018) reported 96.6 % removal of tetracycline at pH 6.0. Dao *et al*. (2018) reported maximum adsorption of cefixime at pH 6.0. At lower pH, antibiotics and beads carry positive charges due to protonation of amine groups on their surface and at higher pH, they behave as anion due to deprotonation of carboxylic groups, however; at neutral pH Zwitter ion exists (Yadav *et al*., 2018). Thus, the present study was spread over with range from pH 3-11. Maximum adsorption of antibiotics were observed at pH 5.0-7.0 on beads, which is also found in a research where in amoxicillin adsorption increases at pH 5 as the carboxyl functional groups (-COOH) on the amoxicillin readily

dissociate to carboxylate (-COO) which increases electrostatic attraction between amoxicillin and the adsorbent (Putra *et al*., 2009; Moussavi *et al*., 2013). The adsorption was also observed to be maximum at pH 7.0 on beads, because of its Zwitter ionic form which may be due to protonated amine groups that is still able to help in adsorption (Yadav *et al*., 2018). Deprotonation of C=O groups were responsible for decrease in adsoprtion on antibiotic and beads, which significantly cause repulsion between negative charges found on beads and antibiotics (Jiang *et al*., 2013). That is the reason adsorption was observed to be decreasing after pH 7.0.

In our studies increase in initial concentration of cefixime, cefotaxime, gemifloxacin and gatifloxacin antibiotics decreased the adsorption efficiency of beads (**Fig. 16d, 19d, 22d, 25d**). The adsorption of cefixime on AWC beads decreased 35.48%, on CAW beads 30.85% and on WAC beads 35.48%. The adsorption of cefotaxime on AWC beads decreased 32.53%, on CAW beads 42.35% and on WAC beads 27.65%. The adsorption of gemifloxacin on AWC beads decreased 31.57%, on CAW beads 26.88% and on WAC beads 27.65%. The adsorption of gatifloxacin on AWC beads decreased 34.37%, on CAW beads 32.29% and on WAC beads 34.04%. The results obtained in this study are in good agreement with the results reported in previous studies. It was reported by Khosravi *et al*. (2018), adsorption percentage of cephalexin decreased from 62.92% and 48.36% onto TiO_2/GLM and GLM respectively with the increase in initial concentration of cephalexin from 20-200 mg/L. Feng *et al*. (2018) reported norfloxacin adsorption decreased from 92% to 77% with the increase in initial concentration from 20-80 mg/L. Fu *et al*. (2019) reported decrease in adsorption from 90%-35% with the increase in initial concentration of ciprofloxacin. With the increase in initial concentration of chlortetracycline antibiotic from 8.8 to 82.2 mg/L, the adsorption was observed to be decreasing from 82.9%-73% (Tunc *et al*., 2020). Liang *et al*. (2018) reported increase in adsorption capacity with the increase in initial concentration on nanoparticles. The percentage efficiency of tetracycline and chlortetracycline was observed to be decreasing with the increase in initial concentration (Tang *et al*., 2018). Wang *et al*. (2019) also reported maximum adsorption capacity of 229.7 and 339.6 mg/L for ciprofloxacin and enrofloxacin on chitosan hydrogels with the increase in initial concentration from 10-600mg/L and adsorption was observed to be decreasing. The adsorption of antibiotics decreased

after the formation of monolayer at lower concentration over the adsorbent surface, which results in saturation of active sites. Formation of the layer at higher concentrations is hampered by the interaction between the antibiotics and the bead surface (Balarak *et al*., 2016).

Chapter - 7

Conclusion

Chapter - 7

CONCLUSION

Antibiotics are the important class of pharmaceuticals used for the management of human and veterinary diseases, inhibit the growth of microorganisms including protozoans, bacteria and fungi. After consumption, these compounds are partially metabolized and are discharged into the aquatic environment. Several researchers have reported the presence of antibiotics in different water compartments. Presently antibiotic accumulation in the aqueous bodies have become a great cause of concern. The presence in the water bodies disturb the ecological stability, affecting the typical activity of animals, microbes, plant life and ultimately human health. Their presence in the aquatic environment have grabbed attention to the potential toxicity on non-target species also. The longtime exposure to low-level antibiotics produce toxic effects which include hypersensitive reactions, abnormalities in digestive system functioning, allergic reactions and the development of antibiotic resistance to the microorganisms.

Conventional technologies have been used to removal the antibiotics from waste waters, but are mostly not able to remove antibiotics efficiently. Among the model organism *Daphnia magna* and *Danio rerio have* been used extensively in regulatory testing and ecotoxicological research. Several features of *Daphnia magna* make it suitable for toxicity testing model. Many characteristics make the zebra fish, such as their ease of manipulation, frequent spawning, external fertilization, large number of offspring, transparent embryos and short generation time makes them an attractive model organism to assess the toxicity of drug-targeted organs and the related mechanisms.

During the current research the significant findings are summarized as below:-

1. The acute toxicity test was performed on *D. magna* neonates in a static exposure for 48h, using modified US, EPA 2002 standard procedures. Among the two cephalosporin and fluoroquinolone antibiotics *D. magna* was more susceptible to cefotaxime (25.82 mg/L) antibiotic.

- The 48h-EC_{50} cephalosporins i.e. cefixime and cefotaxime against *D. magna* was 77.92 mg/L and 25.82 mg/L respectively.
- In case of fluoroquinolone antibiotics the EC_{50} value for gatifloxacin was 330.8 mg/L after 48 hrs and for gemifloxacin was 489.42 mg/L respectively.

2. The chronic toxic effects of antibiotics on *D. magna* were:-

- When treated with cefixime antibiotic the survival of *D. magna* was not significantly inhibited when exposed upto 2.1 mg/L, and survival was observed to be 85 % at the highest antibiotic concentration (56.7 mg/L). There was no significant difference observed in the first day of brood and control. The number of young per female and number of young ones per brood were not affected with the increase in cefixime antibiotic concentration.
- The survival of daphnids significantly reduce with the increase in cefotaxime antibiotic concentration and 50% daphnids survived at the highest concentration (16.2 mg/L). Delay in reproduction was observed after 5.4 mg/L. Number of young per female/ brood were decreased after 0.60 and 1.8 mg/L cefotaxime concentration in the chronic assay.
- In case of gemifloxacin the first day of reproduction was statistically significant till 10.5 mg/L and after 31.5 mg/L the toxic effect of the antibiotic concentration could not be tested since all daphnids die at these concentrations. The average number of neonates per female was constantly decreasing with the increase in concentration when compared with control. The number of young ones per brood decreased after 10.5 mg/L.
- Gatifloxacin antibiotic reduced the survival of daphnids in a dose dependent manner. At the highest gatifloxacin antibiotic concentration i.e. 202.5 mg/L, 100% mortality was observed. The initial concentrations of gatifloxacin had little effect at first day of reproduction and after 67.5 mg/L the toxic effect of the antibiotic concentration could not be tested since all daphnids die at these

concentrations. Comparing with control the average number of young per female were also affected and at highest concentrations (67.5 and 202.5 mg/L) no production of young ones were observed. The number of young per brood significantly decreased at 22.5 mg/L and no daphnid was observed after 67.5 mg/L gatifloxacin concentration.

3. OECD, 2006 guidelines were used for acute toxicity testing of *D. rerio*.

   - The survival of embryos were observed 100% in all concentrations of cefixime and cefotaxime antibiotics. 0% mortality was observed in negative control and internal plate control. There were no lethal effects and other malformations recorded in embryo development.
   - No effects of categorical end points were recorded on either gemifloxacin or gatifloxacin until at 96 hrs. The survival of embryos were observed to be 100% for the control and treatment groups. No lethal and other malformation were recorded in embryo development.

4. The beads were successfully prepared for the antibiotic adsorption through batch adsorption. The materials used for bead preparation were chitosan, walnut shell and almond shell. The suitability of material used for bead preparation can be justified for being having high sorption capabilities, biocompatibility, low cost, ease in availability and non-toxicity.

5. The characterization of beads where done by SEM analysis, FTIR analysis and EDX analysis.

   - FTIR analysis confirms the presence of N-H, OH and CO groups, which are considered as a good choice for the adsorption process.
   - Scanning electron micrographs (SEM) confirms the presence of sparsely scattered fissures and occasional pores which can be helpful in the adsorption of antibiotics.
   - Energy dispersive spectra (EDX) define the elemental peaks of AWC, CAW and WAC beads. The elemental composition of beads contain carbon, oxygen and nitrogen.
   - In our studies we observe adsorption of antibiotics increase with the increase in adsorbent dosage of prepared beads.

- With the increase in initial concentration of antibiotics adsorption decreased due to saturation of the active sites.
- Adsorption equilibrium was obtained within 120 minutes in most of the adsorption studies.
- The maximum adsorption of antibiotics in our studies were observed between pH 5-7.
- The present study revealed that beads could be used as cheap and effective adsorbents for the removal of antibiotics from aqueous solution.

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