ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

DETERMINATION OF NANOTOXICOLOGICAL EFFECTS OF SILVER (Ag^0) AND ALUMINIUM (Al^0) NANOPARTICLES ON MICROBIAL COMMUNITY STRUCTURE IN ACTIVATED SLUDGE

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

GÜMÜŞ (Ag^0) VE ALUMİNYUM (Al^0) NANOPARÇACIKLARININ AKTİF ÇAMURDAKİ MİKROBİYAL YAPIYA OLAN NANOTOKSİKOLOJİK ETKİLERİNİN BELİRLENMESİ

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Elif Buket PARLAK, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 513101004, successfully defended the **thesis** entitled "**DETERMINATION OF NANOTOXICOLOGICAL EFFECTS OF SILVER (Ag^0) AND ALUMINIUM (Al^0) NANOPARTICLES ON MICROBIAL COMMUNITY STRUCTURE IN ACTIVATED SLUDGE**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. İsmail KOYUNCU** Istanbul Technical University	.....
Jury Members :	**Prof. Dr. İsmail KOYUNCU** Istanbul Technical University	.....
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To my family,

FOREWORD

Emerging nanomaterials have a wide range of specific industrial applications including textiles, cosmetics, food packaging and so on. The usage of nanomaterials cause nanoparticles to enter sewage pipes and the wastewater treatment plants (WWTPs). However, studies on inhibition of microbial growth by different nanopaticles in wastewater treatment systems are limited. In this thesis work, the inhibitory effects of two types of nanoparticles on bacterial growth in activated sludge were determined by experiments of short and long term analysis. In addition, respirometric analysis were carried out.

I will first and foremost like to thank my great supervisor Prof. Dr. İsmail KOYUNCU for giving me very good back-up and advices. I am deeply greatful to Dr. Derya Y. İMER for all her tireless support and guidance all the time when I got stuck.

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Finally, special thanks go to my family and close friends for their ever-present support in my life.

"The place to improve the world is first in one's own heart and head and hands, and then work outward from there." ― Robert M. Pirsig

June 2013

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ABBREVIATIONS

NP **:** Nanoparticle
AgNP **:** Silver nanoparticle
AlNP **:** Aluminium nanoparticle
MLSS **:** Mixed liquor suspended solids
SMP **:** Soluble microbial products
EPS **:** Extracellular polymeric substances

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DETERMINATION OF NANOTOXICOLOGICAL EFFECTS OF SILVER (Ag^0) AND ALUMINIUM (Al^0) NANOPARTICLES ON MICROBIAL COMMUNITY STRUCTURE IN ACTIVATED SLUDGE

SUMMARY

Nanoscience and nanotechnology are dynamically developing fields of scientific interests in the entire world. This technology promises scientific advancements in various sectors such as medicine, consumer products, energy, materials and manufacturing. Nanotechnology is somewhat loosely defined, although in general terms it covers engineered structures, devices, and systems that have a length scale between 1 and 100 nanometers. Researching, developing and utilizing are the heart of this new technology. Nanotechnology offers potentially huge benefits to society, industry, environment and health. It helps us to improve quality of our lives and responds some key issues of day as climate change by reducing greenhouse gas emissions. Other potential benefits include contributions in improving energy storage and efficiency, better diagnosis and treatment of disease, faster computer systems and remediation of air, soil or water pollution.

Nanoparticles may be synthesized from several materials by various physical and chemical methods, with particles differing in their elemental composition, shape, size, and chemical or physical properties. At nanoscale dimensions, properties of materials may change significantly to differ completely from their bulk forms. As the size of material decreases, proportion of surface atoms increase, which also increases reactivity and turns them into highly reactive catalysts with the surface atoms of active centers for elementary catalytic processes. Thus, nanoparticles possess unique electronic, chemical, optical, magnetic and mechanical properties that arise explicitly due to their nanometer-scale size. Because of those unique properties, NPs can be employed in applications in various fields, such as catalysis, wastewater treatment, textiles, paints, drug delivery, magnetic resonance imaging (MRI), tissue engineering, and cancer treatment. Multifarious engineered nanoparticles have been at the center of extraordinary and highly focused efforts from both industry and academic studies in recent years. Nanoparticles possess extremely high surface area to volume ratio due to their small sizes, which renders them highly reactive. High reactivity potential could lead to toxicity due to harmful interactions of nanoparticles with biological systems and the environment. Nanotoxicology was proposed as a new branch of toxicology to address the gaps in knowledge and to specifically address the adverse effects likely to be caused by nanoparticles. Discipline of nanotoxicology would make an important contribution to the development of a sustainable and safe nanotechnology. Nanotoxicology encompasses the physicochemical determinants, routes of exposure, biodistribution, molecular determinants, genotoxicity, and regulatory aspects. In addition, nanotoxicology is involved in proposing reliable, robust, and data-assured test protocols for nanomaterials in human and environmental risk assessment. Release of nanoparticles

to the environment during recycling and disposal are particular concerns for nanoparticles incorporated into limited use and/or disposable products. Once those nanomaterials are released, they would readily undergo transformation via biotic and abiotic processes. Understanding environmental transformations and fate of engineered nanoparticles enables design and development of environmentally benign nanoparticles. Number of engineered nanoparticles are increasing day-by-day, and it is expected that the nanoparticles will be more complex and will have unique chemistry; therefore in order to ensure 'safe' nanotechnology, 'nanotoxicology' studies would require a standard set of protocols for their toxicity (including genotoxicity, teratogenecity) and ecotoxicity.

Increasing use of nanomaterials introduces the nanoparticles intentionally/unintentionally into the waste streams and wastewater treatment facilities. The impact that wastewater treatment has on nanomaterials, or conversely, the impact that nanoparticles have on wastewater treatment, is largely unknown. Moreover, questions still remain on the efficient ways to remove those nanoparticles from industrial and domestic wastewater treatment plants. Recent studies suggest that some nanoparticles are not held in treatment plants and are discharged into natural water bodies. Those nanoparticles may remain in the environment for long periods and may be potentially toxic to the aquatic life. Upon release, nanoparticles are likely to interact with aquatic surfaces and biological species as well as aggregate, depending on the interplay between electrostatic and van der Waals interactions. For this reason, there is an urgent need to analyze possible behaviors and fates of nanoparticles in wastewater treatment facilities and wastewater sludge.

This study focuses on identifying relationships between specific nanoparticles and real activated sludge. Therefore, this study investigates by using respiration tests and biological analysis, the inhibitory effects of two different commonly used metal oxide (Ag^0 and Al^0) nanoparticles on the activity of the microbial communities present in a real wastewater treatment plant. Experiments were performed in three different stages. Firstly, dissolution ratios of nanoparticles were determined at different experimental conditions. Secondly, effects of nanoparticles were investigated at short-term toxicity tests and respirometric analysis. Finally, toxicity effects of nanoparticles were determined at long-term tests.

GÜMÜŞ (Ag^0) VE ALUMİNYUM (Al^0) NANOPARÇACIKLARININ AKTİF ÇAMURDAKİ MİKROBİYAL YAPIYA OLAN NANOTOKSİKOLOJİK ETKİLERİNİN BELİRLENMESİ

ÖZET

Nanobilim ve nanoteknoloji, bilimsel çevrelerde dinamik olarak tüm dünyada gelişen alanlardır. Bu teknoloji ilaç, tüketim ürünleri, enerji, malzeme ve imalat alanında bilimsel gelişmeler sunmaktadır. Nanoteknoloji en basit haliyle, genel olarak mühendislik yapılarını kapsasa da 1 ve 100 nanometre arası boyutlar ölçeğinde düzenek ve sistemler olarak tanımlanabilir. Araştırma, geliştirme ve bu özelliklerin kullanımı, yeni teknolojinin merkezinde yer almaktadır. Nanoteknolojinin toplum, sanayi, çevre ve sağlık alanlarında da çok büyük fayda sağlama potansiyeli vardır. Yaşam kalitemizi arttırması ve sera gazı emisyonlarının azaltılması gibi bazı günlük kilit konularda yardımcı olabilmektedir. Enerji depolanması ve veriminin artırımına katkısı, hastalıklara daha iyi tanı ve tedavi yöntemleri sunması, bilgisayar sistemlerini hızlandırması ve kirlenmiş hava, toprak ve suyun iyileştirilmesi gibi diğer kullanım alanları mevcuttur.

Nanopartiküller birçok farklı maddeden, elementel kompozisyonu, şekli, boyutu, fiziksel ve kimyasal özellikleri değişim göstererek, çeşitli fiziksel ve kimyasal metotlarla sentezlenebilirler. Nanoboyutta maddelerin özellikleri yığın formuna göre belirgin şekilde değişim gösterir. Maddenin boyutu küçüldükçe atom yüzey alanı artar, bu da reaktifliği arttırır ve yüzeydeki atomlar basit katalitik proseslerin aktif merkezleri olmasıyla yüksek reaktif katalistler haline dönüşürler. Böylece nanopartiküller nanometre ölçeği boyutunda benzersiz elektronik, optik, manyetik ve mekanik özellikler gösterirler. Bu benzersiz özellikleri sebebiyle nanopartiküller kataliz, atıksu arıtımı, tekstil, boya, ilaç iletimi, manyetik rezonans görüntüleme (MRI), doku mühendisliği ve kanser tedavisi alanlarında kullanılmaktadır. Farklı tipteki nanopartiküllere son yıllarda hem sanayi hem de akademik çalışmalarda yoğun şekilde odaklanılmıştır. Nanopartiküller çok küçük boyutlardaki hacimlerine göre geniş yüzey alanları sebebiyle çok reaktiftirler. Yüksek reaktivite potansiyeli, nanopartiküllerin biyolojik sistem ve çevreyle zararlı ilişkisine bağlı olarak toksisiteye sebep olma potansiyeline sebep olabilmektedir. Nanotoksikoloji, özellikle nanopartiküllerin neden olduğu zararlı etkiler alanındaki bilgi boşluğunu doldurmak amacıyla toksikolojinin yeni bir dalı olarak gelişmektedir. Nanotoksikoloji disiplini, sürdürülebilir ve güvenli bir çevrenin gelişimi için önemli katkılar sağlamaktadır. Nanotoksikoloji, fizikokimyasal bileşenler, maruz kalma yolları, biyodağılım, moleküler bileşenler, genotoksikoloji, ve yasal denetimler konularını kapsamaktadır. Ek olarak, nanotoksikoloji nanomateryallerin insan ve çevresel risk değerlendirilmesinde güvenilir, sağlıklı, ve doğruluğundan emin olunan test yöntemlerini de içine alır.

Limitli kullanımı olan ürünlerde bulunan nanopartiküllerin, kullanım sonrası bu ürünler uzaklaştırıldığında nanopartiküllerinin geri dönüşümü ve uzaklaştırılması

sırasında çevrede yayılımı merak konusudur. Bu nanomateryaller bir kez yayıldıktan sonra biyotik ve abiyotik proseslerle değişime uğrayabilirler. Çevresel dönüşümleri ve işlenmiş nanopartiküllerin zararlarının anlaşılabilmesi nanopartiküllerin tehlikesiz uzaklaştırılmasının tasarım ve gelişimine olanak sağlayacaktır. Kullanılan nanopartikül sayısı günden güne artış göstermektedir, ve bu da nanopartikülleri daha kompleks yapmakta ve benzersiz kimyasal özellikler kazandırmaktadır; bu sebeple 'güvenli' nanoteknoloji için, 'nanotoksikoloji' çalışmaları toksikoloji (genotoksikoloji ve teratogenitik) ve ekotoksikoloji için standart protokollerin getirilmesi gerekmektedir.

Nanomateryallerdeki kullanım artışı, nanopartiküllerin kasıtlı/kasıtsız olarak kanalizasyon ve atıksu arıtma tesislerine girmesine sebep olmaktadır. Atıksu arıtımının nanomateryaller üzerindeki etkileri veya bunun tersi nanomateryallerin atıksu arıtım tesisi üzerindeki etkileri bilinmemektedir. Dahası, bu maddelerin endüstriyel veya evsel atıksu arıtma tesislerinden verimli uzaklaştırılmasının yolu konusunda da soru işaretleri vardır. Son çalışmalar nanopartiküllerin atıksu arıtma sisteminde tutulamadığını ve doğal su ortamlarına deşarj edildiğini göstermektedir. Bu nanopartiküller doğada uzun süre kalmakta ve su yaşamında potansiyel toksik etki göstermektedir. Nanopartiküller yayıldıkları zaman, elektrostatik ve van der Waals etkileşimleri ile sulak yüzeylerle etkileşmekte ve biyolojik türlerle birleşmektedir. Bu sebepten dolayı, atıksu arıtma tesisleri ve atıksu çamurundaki nanopartiküllerin olası davranış ve zararları üzerine yeni analiz yöntemleri gerekmektedir.

Bu çalışma, belirli nanopartiküller ile gerçek aktif çamur arasındaki ilişkinin karakterinin belirlenmesi üzerinedir. Bu sebeple bu çalışmada, endüstriyel bazda yaygın olarak kullanılan iki farklı metal oksit (Ag^0 ve Al^0) nanopartikülünün, gerçek atıksu arıtma tesisinde bulunan mikrobiyal toplulukların aktivitesi üzerindeki inhibisyon etkileri, solunum testleri ve biyolojik analizler ile araştırılmıştır. Deneyler üç aşamada gerçekleştirilmiştir. İlk olarak farklı deneysel koşullarda nanopartiküllerin çözünme oranları belirlenmiştir. İkinci olarak kısa süreli toksisite testleri ve solunum analizleri ile nanopartiküllerin etkisi çalışılmıştır. Son olarak ise uzun süreli testlerle nanopartiküllerin toksik etkisi belirlenmiştir.

Nanopartiküllerin çözünme deneylerinde pH (3 - 5 - 7), sıcaklık (25^oC – 35^oC – 45^oC), karıştırma hızı (200 rpm – 400 rpm – 600 rpm – 800 rpm), nanopartikül konsantrasyonu (0.1 - 0.2 - 0.6 - 1%) parametreleri değişimine bağlı olarak farklı deneysel koşullarda analizler gerçekleştirilmiş ve bu numunelerin ICP ölçümleri yapılmıştır. İlk olarak yapılan pH deneyinde sıcaklık, karıştırma hızı ve nanopartikül konsantrasyonu sabit tutulmuş, optimum pH değeri seçilmiştir. İkinci yapılan sıcaklık testinde karıştırma hızı sabit tutulmuş, optimum pH ayarlanmış ve farklı sıcaklılarda yapılan analizler sonucu optimum sıcaklık değeri seçilmiştir. Daha sonra optimum pH ve optimum sıcaklık değerlerinde sabit nanopartikül konsantrasyonunda farklı karıştırma hızları arasında optimum karıştırma hızı seçilmiştir. Son olarak da tüm değişkenler optimum değerlerinde sabit tutularak farklı nanopartikül konsantrasyonları denenmiş ve optimum konsantrasyon seçilmiştir. Deneylerin bundan sonraki aşamalarında bu bölümde seçilen optimum değerler kullanılmıştır.

Deneylerin ikinci aşamasında nanopartiküllerin aktif çamur içine kısa süreli şok yüklemesinin biyolojik etkisinin belirlenmesi amaçlanmıştır. Deneyler, optimum koşullar olan pH 7.0, sıcaklık 25.0^0C, karıştırma hızı 400 rpm seçilerek üç farklı nanopartikül oranında (0.05 - 0.1 - 0.2 %, m/m) gerçekleştirilmiştir. Çözünürlük

oranları analizindekilerden farklı nanopartikül oranı kullanılmasının nedeni en yüksek konsansantrasyona 0.1 - 0.2 %, m/m konsantrasyonlarında ulaşılması ve daha düşük konsantrasyonda nanopartikülün etkisinin de araştırılmak istenmesidir. Kısa süreli şok yükleme deneyleri Ag^0 ve Al^0 nanopartikülleri için üçer saat süreyle birer saat periyotlarla numune alınarak gerçekleştirilmiştir. Deney başlangıcında alınan numunenin askıda katı madde miktarı ve SMP-EPS analizleri yapılmış, diğer saatlerde alınan numunelere yalnızca $SMP_{protein}$, $SMP_{karbonhidrat}$, $EPS_{protein}$, $EPS_{karbonhidrat}$ analizleri gerçekleştirilmiştir.

Deneylerin üçüncü aşamasında nanopartiküllerin uzun süreli (10 gün) etkileri incelenmiştir. Bu aşamada nanopartikül konsantrasyonlarına bağlı olarak bakterilerin biyolojik etkileri üzerine etkisi incelenmesi amaçlanmıştır. Şok yükleme deneylerinde kullanılan aynı deneysel şartlar kullanılmış fakat kısa süreli deneylerden farklı olarak aktif çamurlar sabit hızlı difüzörler ile havalanmıştır. Kısa süreli şok yükleme analizinde verilen toplam nanopartikül miktarı 10 gün süresince 24'er saatlik periyotlarla parça parça aktif çamura verilmiştir. Bu deneyler süresince günlük olarak AKM, viskozite, $OD_{çamur}$, $OD_{süpernatant}$, $SMP_{protein}$, $SMP_{karbonhidrat}$, $EPS_{protein}$, $EPS_{karbonhidrat}$ analizleri yapılmıştır. Bu deneylerde çamurun AKM, viskozite, $OD_{çamur}$, $OD_{süpernatant}$, $SMP_{protein}$, $SMP_{karbonhidrat}$, $EPS_{protein}$, $EPS_{karbonhidrat}$ değerleri analiz edilmiş, bir gece besleme yapılmamış, daha sonra sentetik evsel atıksu ve nanopartikül beslemesi yapılarak deneyler gerçekleştirilmiştir.

Solunum hızı deneylerinde nanopartikülün miktarına bağlı olarak değişen içsel solunum hızı ve besin varlığındaki solunum hızı üçer saat süresince onar dakikalık periyotlarla ölçülerek analiz edilmiştir. Kısa süreli şok yükleme ve nanopartiküllerin uzun süreli etkileri deneylerinde olduğu gibi 0.05 - 0.1 - 0.2 %, m/m nanopartikül konsantrasyonları ile nanopartikül eklenmiş numuneler üzerinde deneyler gerçekleştirilmiştir. Bir gece sadece havalandırılarak aç bırakılan numunelerin havası kesilmiş ve çözünmüş oksijen probu ile içsel solunum hızı analizleri gerçekleştirilmiştir. Daha sonra glikoz eklenen numunelerin üçer saat boyunca onar dakika arayla çözünmüş oksijen miktarları ölçülmüştür. Bu analiz sonucu bulunan değerlerden içsel solunum hızları çıkarılmış ve substrat varlığında oksijen kullanım oranları bulunmuştur. Böylece nanopartikül konsantrasyonlarının değişim oranlarına göre nanopartikülün toksik etkisi bakteriyel inhibisyon oranına bağlı olarak bulunmuştur.

Yapılan analizlerin sonucu olarak AgNP'ünün pH 7'de en fazla çözünürlüğü gösterdiği, sıcaklık artımı ile AgNP çözünürlüğünün azaldığı, karıştırma hızı olarak 400 rpm'de maksimum çözünürlüğe ulaşıp daha sonra düşüşe geçtiği, %0.1 konsantrasyon oranında maksimum çözünürlüğe sahip olduğu gözlenmiştir. Bu ölçüm sonuçlarına göre sonraki aşamalardaki AgNP konsantrasyonları ve ortam şartları seçilmiştir. AlNP ise AgNP ile hemen hemen paralel ancak daha az oranda değişim göstermesi nedeni ve deneylerin gözlenebilirliği açısından aynı şartlarda uygulanmasına karar verilmiştir ancak AlNP için 200 rpm karıştırma hızı kullanılmıştır. Çözünen nanopartikül konsantrasyonu oranının çok düşmesi sebebiyle %0.6 ve %1.0 oranları yerine %0.05 oranı ile çalışmalara devam edilmiştir.

İkinci aşama olan kısa süreli şok yükleme deneylerinde AgNP'nin SMP_p üzerine etkisi incelendiğinde %0.05 konsantrasyonunda kayda değer bir etki gözlenmemiş, %0.1'de bir artış ve %0.2'de ikinci saat pik değeri sonrası başlangıç seviyesine yaklaşma gözlenmiştir. SMP_c üzerinde etkisi olmadığı görümüştür. EPS_p için ise % 0.05 için belirgin bir artış görülmüş olmasına rağmen diğer konsantrasyonlarda sabit

seyir gözlenmiştir. EPS_c oranı ise AgNP eklenmesiyle belirgin bir artış göstermiştir. AlNP eklendiğinde ise SMP_p oranı ilk saat yükselmiş olmasına rağmen daha sonra başlangıç değerlerine dönmüştür, SMP_c için ise bir değişim gözlenmemiştir. EPS_p için ise %0.05 ve %0.1 konsantrasyonlarında azalış, %0.2 oranında artış görülmüştür. EPS_c için AlNP'nın etkisi araştırıldığında ise %0.05 için artış, diğer oranlar için ise başlarda çok az bir artış görülmesine rağmen daha sonra başlangıç değerlerine yaklaşmıştır.

Respirometrik deneylere bakıldığında ise AgNP AlNP'ye oranla daha fazla olmak üzere içsel solunum hızını azalttığı gözlemlenmiş, ve nanopartikülün bakteri üzerinde inhibisyon etkisi olduğu sonucuna varılmıştır.

Üçüncü aşama olan nanopartiküllerin aktif çamur üzerindeki uzun süreli etkisinde AgNP için bakteriyel büyümeyi belirgin şekilde etkilemediği, viskozite değerini değiştirmediği, OD üzerinde izlenebilen bir etkisi olmadığı görülmüştür. AgNP konsantrasyonun artması SMP_p ve SMP_c konsantrasyonunu da arttırmıştır. AgNP eklenmesiyle EPS_p azalmış, fakat EPS_c benzer salının göstermiştir. $OD_{supernatant}$ sonuçları ele alındığında AgNP içermeyen çamurun çökelme özelliğinin daha iyi olduğu görülmektedir. AlNP ile yapılan analizlerde ise bakteriyel büyümenin azaldığı,viskozitenin ve $OD_{çamur}$'un değişmediği görülmektedir. SMP_p değişimi ise ilk iki gün artış gösterdikten sonra dengelendiği, yedinci günden sonra ise AlNP içerenlerin SMP_p konsantrasyonu azalırken nanopartikül içermeyen çamurun SMP_p konsantrasyonunun arttığı görülmektedir. %0.05 AlNP konsantrasyonunda SMP_c en yüksek değerde okunurken, nanoparçacık içermeyen çamurda bu değer en düşük olarak okunmaktadır. EPS_p ve EPS_c konsantrasyonlarının ise AlNP eklenmesiyle arttığı gözlenmektedir. $OD_{supernatant}$ değerinin nanopartikül içermeyen dahil tüm çamurlarda arttığı görülmektedir, buna rağmen, %0.1 AlNP konsantrasyonunda en yüksek değerler gözlenmiştir.

1. INTRODUCTION

At the annual meeting of the American Physical Society on December 29, 1959 at the California Institute of Technology, a talk is given which was titled "There's Plenty of Room at the Bottom" that has become a classic in 20th century science lectures by Nobel Laureate Richard P. Feynman. A few years before the word "chip" has taken place in dictionaries, Feynman demonstrated extrememiniaturization in 1959. Because of enormously small structures of biological systems, Feynman was inspired by biology. He said, "Many of the cells are very tiny, but they are active; they manufacture substances; they walk around; they wiggle; and they do all kind of marvellous things–all on a very small scale.". Also, they store information. Since that talk, nanotechnology is developing day by day, which reflects our lives as nanomaterials and nanoproducts from food through electronics, in other words all parts of our lives more and more every single day. All of those products complete their life time and return to the environment. The signifcant increase in the production and application of engineered nanoparticles inevitably result in the release of these materials into the natural environment. For many engineered nanoparticles, sewage and industrial discharges are the primary pathways of release. The widespread usage of products containing nanoparticles would certainly release these nanoparticles into sewer systems and subsequently into municipal wastewater treatment plants.

Wastewater treatment plants basically consist of 3 parts. The first stage which is called as primary treatment, is responsible for physical treatment, where coarse matters are filtered in coarse screens, oil, grease and other floating material is removed from surface, and settled heavy solids are removed from bottom. The second stage is secondary treatment, where dissolved and suspended biological matter is removed. The secondary treatment unit is the habitat of microorganism which degrade biological content of sewage such as feces, food waste, soaps and detergents, put another way major contents of a domestic wastewater. The last part of

a wastewater treatment plant is tertiary treatment which provides a final treatment before discharge to improve the effluent quality.

Antibacterial activities of NPs are reported in many studies, however, toxic effects of those particles are still not investigated very well. Impacts of NPs on ecosystem can be understood by researching interactions between microorganisms and NPs, as studying on test model single cell structure of the microorganisms lead to provide information about how NPs impact on cell/organism function.

1.1 Purpose of Thesis

The main objective of this study is to investigate the nanotoxicologic effects of silver nanoparticles (AgNPs) and aluminium nanoparticles (AlNPs) at lab-scale real activated sludge medium. As described above, in a wastewater treatment facility, main degradation is biodegradation which is performed by microorganisms at activated sludge. This study focuses on the toxicological effects of AgNPs and AlNPs on these microorganisms. The study presents the nanotoxicological effects of AgNPs and AlNPs due to being manufactured at the industrial level and having numerous applications. AgNPs are the most commonly used NPs within approximately 30% of such commercial products due to their antimicrobial properties. Due to its properties there is a great potential for the use of NPs in numerous products such as medical devices and supplies, food packaging materials and food supplements, odour-resistant textiles, electronics and household appliances, cosmetics, water disinfectants, and room sprays. AlNPs have been found a wide application area for last ten years due to their high effective catalysis and improved sintering processes properties. AlNPs are mostly used in dispersion-strengthening, nanocomposites, catalyst support, transparent conductive coatings, biomaterials, heat-transfer fluids (suspensions), drug delivery, sources for IC board or package, transparent optical coatings, wear-resistant additive, material surface coatings. Increase in such products results in increased load of NPs in wastewater treatment plants.

1.2 Scope of Thesis

This study was carried out by using two different metallic nanoparticles (AgNPs and AlNPs). The aim is to find out the interactions between nanoparticles and microbial medium. Experiments were carried out together with the dissolution properties of nanoparticles and microbiological characterization showed important results in terms of nanotoxicological effects of AgNPs and AlNPs in activated sludge. Comprehensive microbiolgical tests of activated sludge provided the data for determination of both short-term and long-term nanotoxicological effects. Inhibitory effects of those nanoparticles were also determined .

2. LITERATURE REVIEW

2.1 Defining Nanotechnology and Nanomaterials

Nanotechnology term was firstly used to mention the ability of engineer materials precisely at the nanometer level in 1974 by Taniguchi from University of Tokyo. (RSRAE, 2004). However, making an extensive definition of nanotechnology was not very easy. U.S. National Nanotechnology Initiative (NNI) has applied one of the most cited definitions of nanotechnology as follows: "Nanotechnology is the understanding and control of matter at dimensions of roughly 1 to 100 nanometers, where unique phenomena enable novel applications. At this level, the physical, chemical, and biological properties of materials differ in fundamental and valuable ways from the properties of individual atoms and molecules or bulk matter" (NSETS, 2004). Similarly, the American Society for Testing and Materials International defines nanotechnology as "A term referring to a wide range of technologies that measure, manipulate, or incorporate materials and/or features with at least one dimension between approximately 1 and 100 nanometers (nm). Such applications exploit the properties, distinct from bulk/macroscopic systems, of nanoscale components" (ASTM Int'l. 2006).

In 2004, the Royal Society and Royal Academy of Engineering did a clear distinction for the definitions of "nanoscience" and "nanotechnologies". Nanoscience was defined as "the study of phenomena and manipulation of materials at atomic, molecular and macromolecular scales, where properties differ significantly from those at a larger scale", although nanotechnologies were defined as "the design, characterization, production and application of structures, devices and systems by controlling shape and size at nanometer scale." (RSRAE, 2004)

To define a system or material as related to nanotechnology, two criteria should acquire. First of them is that one of its dimensions must be in the approximate range of 1-100 nm, and the other one is that dissimilar properties than the bulk form must

be given to the system by this nanostructure. However, a material or system can be particularly established whether it is related with nanotechnology or not (Hansen et al., 2007).

The nanomaterials are categorized according to the location of the nanostructure in the material. Hansen et al. (2007) propose a categorization of nanomaterials depending on their place of residence in the system (Figure 2. 1). According to that, materials are divided into three main categories as materials, which are nanostructured in the bulk, materials that have nanostructure on the surface and, materials that contain nanostructured particles. Additionally, those categories can be divided into subcategories. The bulk is divided into two subcategories as the first consists only of one type of material whereas the second consists of two or more different constituents. In the second category, the nanostructure is on the surface. The surface can be built up of the bulk material's nanoscale form or covered un-patterned film of nanoscale thickness on a substrate of a different material, or consisted of patterned film on a substrate, where the film is either nanoscale in thickness, or the pattern has nanoscale dimensions along the surface. Third category contains particles at least two dimensions are nanosized, which can be defined as free nanostructures. Nanostructured particles can have various forms and shapes such as quantum dots, fullerenes, nanotubes and nanowires (Maynard & Aitken, 2007). The last main category, nanoparticles, is divided into four subcategories according to the environment around them. In the first one, nanoparticles bound to the surface of another solid structure; in the second one, nanoparticles are suspended in a liquid; in the third one nanoparticles are suspended in solids; and the last subcategory consists of airborne nanoparticles (Hansen, 2009).

2.2 Development of Nanotechnology and Application Areas of Nanomaterials

Nanotechnology can be an entirely new developing field for human, however, as Feynman mentioned his inspiration from biology in his talk, there are many objects and processes in nature that function on micro to nanoscale for thousands of years. Understanding and limiting these functions enables scientists to produce nanodevices and nanomaterials. European Patent Office and The United States Patent and Trademark Office (USPTO) give patents under nanotechnology class since 2011 and since 1992, respectively. According to European Patent Office, approximately 14734

results found in the worldwide database under nanotechnology class since the beginning of 2011 (European Patent Office).

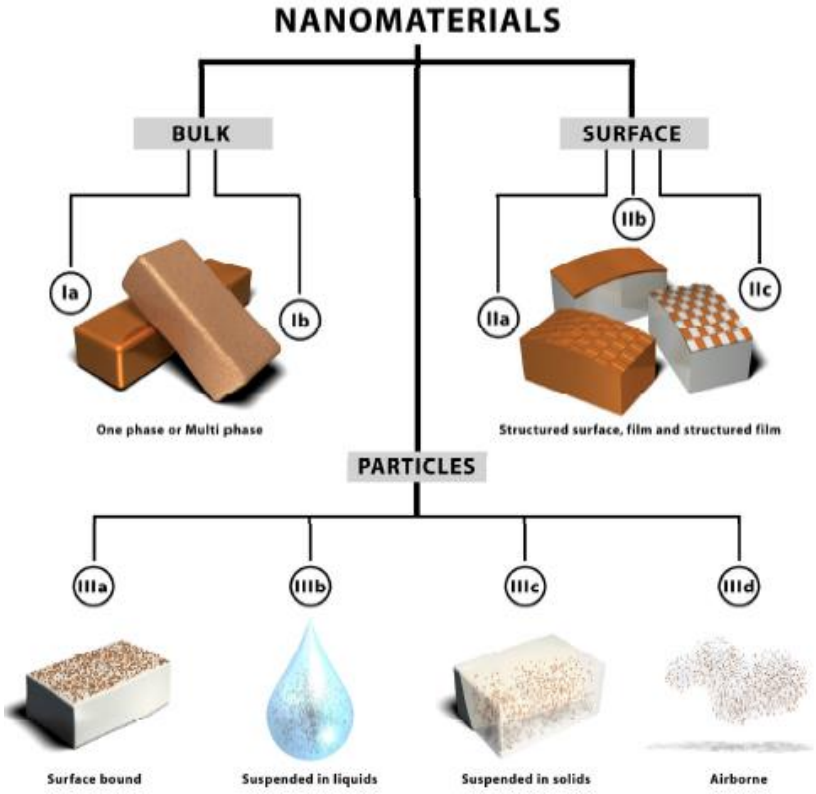


Figure 2. 1 : The categorization framework for nanomaterials (Hansen et al. 2007).

Moreover, Figure 2. 2 shows the number of patents given under nanotechnology class by USPTO;

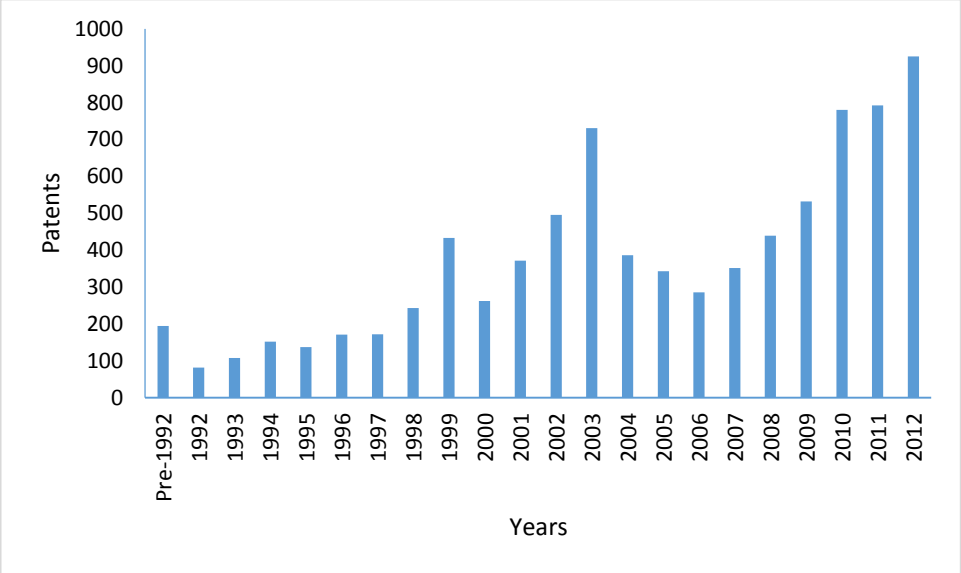


Figure 2. 2 : Number of patents given under nanotechnology class by USPTO (USPTO, 2013).

Currently, many products such as fillers, opacifiers, catalysts, pharmaceuticals, lubricants, cosmetics, pharmaceuticals, electronic devices or other domestic appliances are being included nanoparticles (Nel et al, 2006). Those nanoparticles can be fixed on a substrate or are used in their free form (Piotrowska et al., 2009). Nanoparticles are the most widely used in following sectors; computer, construction, cosmetics, energy, environment, food and drink, medical, packaging, paints and coating, sports and leisure, textiles, clothing and transportation. The most commonly used nanoparticles are Ag, Fe, Pt, Sn, Al, Cu, Zr, Se, Ca, Mg, TiO_2, ZnO, CeO_2, SiO_2, Al_2O_3, carbon black, CNT, fullerenes, nanoclay, ceramic, quantum dots, organic NPs. The application areas of those nanomaterials are given at Table 2. 1.

Table 2. 1 : The application areas of typical nanomaterials (Satinder et al, 2010).

Type pf NPs	**Quantity used in terms of tons**	**Application/Uses**
Ag	High	Antibacterials, medical devices and supplies, food packaging materials and food supplements, odour-resistant textiles, electronics and household appliances, cosmetics, water disinfectants, and room sprays
Fe	High	Water treatment
Pt group metals	High	Catalysts
Sn	Unknown	Paints
Al	High	Dispersion-strengthening, nanocomposites, catalyst support, transparent conductive coatings, biomaterials, heat-transfer fluids (suspensions), drug delivery, sources for IC board or package, transparent optical coatings, wear-resistant additive, material surface coatings
Cu	Unknown	Microelectronics
Zr	High	
Se	Low	Nutraceuticals, health supplements

Table 2. 1 (continued): The application areas of typical nanomaterials (Satinder et al, 2010).

Type pf NPs	Quantity used in terms of tons	Applications/Uses
Ca	Low	Nutraceuticals, health supplements
Mg	Low	Nutraceuticals, health supplements
TiO_2	High	Cosmetics, paints, coatings
ZnO	Low	Cosmetics, paints, coatings
CeO_2	High	Fuel catalyst
SiO_2	High	Paints, coatings
Al_2O_3	Low	Usually substrate bound, paintings
Carbon black	High	Substrate bound, but released with tyre wear
CNT	Medium - High	Used in a variety of composite materials
Fullerenes	Medium - High	Medical and cosmetics use
Nanoclay	High	Plastic packaging
Ceramic	High	Coatings
Quantum dots	Low	Different compositions
Organic NPs	Low	Vitamins, medicines, carriers for medicines and cosmetics, food additives and ingredients

Some of the nanoparticles are used to increase physical properties of materials by adding into their bulk forms. For instance, to strengthen and monitor concrete, carbon nanotubes are used. On the other hand, to improve mechanical properties of the concrete, nano silica can be used which densifies the micro and nanostructure. Furthermore, addition of haematite (Fe_2O_3) nanoparticles to concrete, which enables monitoring stress levels through the measurement of section electrical resistance. Thus, increase in its strength can be observed (Mann, 2006). According to Gao et al. (2007), magnetic nanoparticles have catalytic and magnetic properties. In biochemical tests such as ELISA, magnetic nanoparticles are used. In electrotechnology, acoustics and biochemical analysis ferrofluidic nanoparticles are used (Piotrowska et al., 2009).

Some of the nanoparticles are used to enable materials to gain antibacterial properties. It has known for many centuries that silver has biocidal properties. Because of that, in the production of cutlery and crockery, silver was used for the prevention of bacterial and mould growth. Moreover, due to biocidial properties of silver, still there is an increasing trend for the usage of it in various products (Silver Institute, 2001; Blaser et al., 2008). According to Samsung website, their fridges include silver nanoparticles to decrease the growth of saprophytic bacteria and fungi. Similar to silver nanoparticles, copper nanoparticles have the property to prevent the bacterial growth, will enable a decrease in prices of nano-biocides in the followiıng years (Yoon et al., 2007).

Titanium dioxide (TiO_2) nanoparticles can be used to protect glazing through its sterilising and anti-fouling properties (Meyer et al., 2004). Besides, hydrophilic characteristic" of TiO_2 provides formation of sheets out of rain drops to wash off dirt particles (Mann, 2006). Additionally, nanocrystalline TiO_2 and ZnO are activated by light which have a role in degradation of izothiazoline-3-ones which is a poisonous component in paints. Addition of nanocrystalline TiO_2 and ZnO inhibits the growth of mould and fungi (Kandavelu et al., 2004).

Contaminated groundwater can be purified cost effectively by nanoscience and nanoengineering (Biswas & Wu, 2005). Concentration of toxic components such as metal ions, radionuclides, organic and inorganic compounds, as well as bacteria and viruses, can be decreased up to sub-ppb by usage of nanoparticles (Savage &Diallo, 2005). For the removal of arsenate, bisphenol A and other toxic metals such as Pb^{2+}, Cu^{2+} and Cd^{2+}, carbon nanotubes are used (Peng et al., 2005, Li et al., 2003, Cai et al., 2003). For removal of many harmful agents present in the environment and for waste remediation, magnetite (Fe_3O_4) nanoparticles coated with mesoporous silica is used (Wu et al., 2004). PCBs (polychlorinated biphenyls), organochlorine pesticides and chlorinated organic solvents can be removed from ground water by usage of iron nanoparticles (Zhang, 2003). Polyaromatic hydrocarbons (PAHs) contaminants can be removed with amphiphilic polyurethane (APU) nanoparticles from soils (Kim et al., 2003).

Piotrowska et al., (2009) found that the surface properties of nanoparticles, such as hydrophilicity or hydrophobicity, can enhance separation of many pollutants. Catalytic nanoparticles enhance the rates of chemical reactions, thus, they can be relevant for environmental remediation. The reason of that is, nanoparticles have a

greater surface area that provides a higher catalytic activity. Arviddson et.al (2012) explained that, the most of these nanomaterials are currently in the embryonic phase, although some of them, such as silver nanoparticles used for antibacterial purposes and titanium dioxide nanoparticles used in sunscreen, may already have entered the growth phase.

Producing smaller, lighter and faster materials has been a growing trend for many years. Consequently, existing products and processes may be turned into more effective and accordingly, desire less raw materials and energy. For this reason, the nanotechnology in military, space and security fields has high application potentials, IT, electronic and energy industries (Helland, 2004).

In the Figure 2. 3, it can be seen how much nanotechnology is or have potential to take place in our daily lives.

1 - Organic Light Emitted Diodes (OLEDs) for displays
2 - Photovoltaic film that converts light into electricity
3 - Stratch-proof coated windows that clean themselves with UV
4 - Fabrics coated to resist stains and control temperature
5 - Intelligent clothing measures pulse and respiration
6 - Bucky-tubeframe is light but very strong
7 - Hipjoiınt made from biocompatible materials
8 - Nanoparticle paint to prevent corrosion
9 - Thermo-chromic glass to regulate light
10 - Magnetic layers for compact data memory
11 - Carbon nanotube fuel cells to power electronics and vehicles
12 - Nano-engineered cochlear implant

Figure 2. 3 : Potential uses of nanotechnological materials (Twist, 2004).

2.3 Characteristics of Nanoparticles

Physicochemical properties of nanoparticles and synthesis and processing of nanomaterials are described briefly in following.

2.3.1 The physicochemical properties of nanoparticles

Various differences may be observed in physicochemical properties when particles transform from micro to nanoscales. Nanoparticles have two key features that induce their difference in optical, electrical, magnetic, chemical and mechanical properties from bulk forms. Those factors are prevailed size-range quantum effects and increased surface area to volume ratio (Holister et al., 2003). Beside those two major properties, materials in nano form have different physicochemical characteristics as charge, surface hydrophobicity and redox activity than their bulk form (Oberdörster, 2008). As long as the particle is getting smaller, where the surface area to volume ratio increases, a raise in dominance of the behavior of atoms on the surface of a particle over that of those in the interior of the particle is observed. Isolation and interaction with other material properties are affected by that. Higher surface area is also one of the important features for efficiency of catalysis and structures like electrodes, which permits an increase in performance of fuel cells and batteries. However, according to Helland (2004), "higher surface area reduces resource usage in catalytical processes and hence decrease the amount of waste." Furthermore, the expanded surface area of nanoparticles provides additional properties such as strength and/or chemical/heat resistance improvement that the outcome of interactions between the intermixed materials in nanocomposites (Holister et al., 2003).

There is not a gradual increase in quantum mechanical behavior as the particle size decreases. When particle size reaches a certain scale, quantum mechanical behaviors are started to be shown. Moreover, whether the dimensions of nanoparticles decrease below a critical wavelength of light, it renders them transparent. That property makes them appropriate for applications in packaging, cosmetics and coatings (Holister et al., 2003).

A number of other properties of nanoparticles are observed independently beside the influence of surface or quantum effects. For instance, perfectly formed silicon nanospheres, which range in between 40-100 nm diameters, take place between saphire and diamond that are known as the hardest materials (Holister et al., 2003).

2.3.2 Nanomaterial synthesis and processing

It is important to remember that the size of the nanomaterials are a billionth of a meter. To synthesize nanomaterials, both bottom up and top down approaches are used (Figure 2. 4). These techniques work as assemble atoms together or dis-assemble (break or dissociate) bulk solids into finer pieces (Hoffman, 2005).

According to Hoffman (2005), in the top down methods, bulk form is turned into powder form, and then, it is turned to nanoparticles. In the bottom up methods, atoms are turned into clusters, and clusters are turned into nanoparticles. According to British Standarts Institution (2011), 24 different methods are defined as nanoparticle synthesis method. These methods can be classified under top down and bottom up approaches. In the following table definitions of these techniques are given (Table 2. 2);

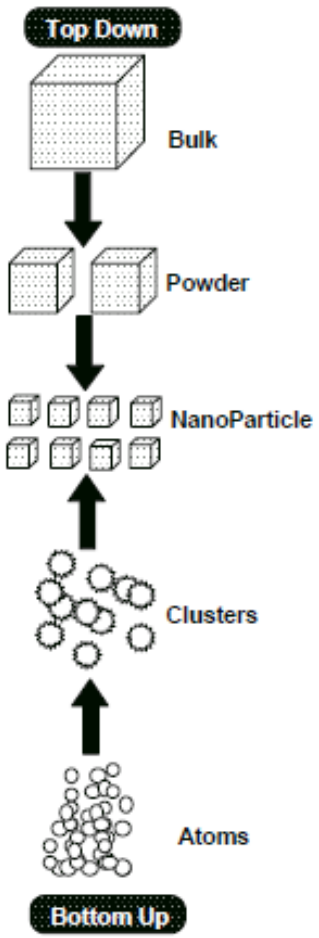


Figure 2. 4 : Schematic representation of the bottom up and top down synthesis processes of nanomaterials (Hoffman, 2005).

Table 2. 2 : The general using techniques for nanoparticle synthesis (British Standarts Institution, 2011).

Techniques	Descriptions
Atomization	Technique used for producing solid particles from a molten material, solution or suspension by spraying under conditions such that it breaks down and then solidifies or dries as a finely divided powder or aerosol
Attrition	Form of comminution, where reduction in size is caused by erosion resulting from the collision of particles with other particles or surfaces
Calcination	Producing or modifying powder by heating to a high temperature in a dry environment
Chemical vapour synthesis	Gas phase synthesis where vapour is formed in a reaction chamber and condenses to form particles
Colloidal production method	Wet chemistry precipitation process in which solutions of different ions are mixed under controlled conditions of temperature and pressure to form insoluble precipitates, which can remain in liquid suspension for distribution and use, or can be used as slurries or collected by filtering or spray drying to produce a dry powder
Comminution	Reduction of particle size by fracture
Electro-explosion	Process whereby a wire is fed into a reactor, and subjected to a high current, high-voltage microsecond pulse to cause it to explode and produce particles
Electrostatic spray assisted vapour deposition	Production method involving spraying atomized precursor droplets across an electric field where the droplets undergo combustion and chemical reaction in the vapour phase
Flame pyrolysis	Synthesis method where flame heat is used to vaporize feedstock Material and initiate chemical reaction to produce particles
Fluidized bed processing	Fabricating or coating with another material within a reactor that uses a suspension of particles in an upward flow of fluid

Table 2. 2 (continued) : The general using techniques for nanoparticle synthesis.

Techniques	Descriptions
Functionalization	Attachment of chemical functional groups to a surface
Furnace flow processing	Gas phase synthesis that produces particles from a saturated vapour for substances having a high vapour pressure at intermediate temperatures
Gas phase synthesis	Production method based on nucleation of a supersaturated vapour and subsequent particle growth by condensation, coagulation and coalescence
High energy milling	Form of attrition production that relies on the use of high levels of kinetic energy to break down material into finer sizes
Laser ablation processing	Physical vapour synthesis using the energy from a (typically pulsed) laser beam to evaporate material from the surface of a target
Laser pyrolysis Molecular self assembly	Gas phase synthesis where a flowing reactive gas is heated rapidly with a laser beam process that produces stable structures by spontaneous assembling of molecules, driven by minimization of gibbs free energy
Physical vapour synthesis (pvs)	Gas phase synthesis in which the vapour is produced by evaporation of a source material and the condensed particles have the same chemical composition as the source
Plasma processing	Plasma processing phase synthesis using a plasma reactor to deliver the energy required to cause evaporation or initiate chemical reactions
Sol-gel processing	Production process involving the conversion of a sol to a gel, which is then desiccated to produce particles
Solution phase templating	Method for producing well defined structures in solution using molecular self assembly in conjunction with a template
Sonication	Physical method to aid the dispersion of nanoparticles in liquid by use of high frequency sound waves

Table 2. 2 (continued) : The general using techniques for nanoparticle synthesis.

Techniques	Descriptions
Sonochemistry	Colloidal production method controlled by acoustic cavitation generating high temperatures and pressures within highly localized regions in a liquid, where molecular precursors undergo chemical reactions due to the application of ultrasound
Thermal spraying and coating	Process for creating particles and coatings using a powder or wire that is partially melted using gas or plasma flames

2.4 Nanotoxicology

Society of Toxicology defines toxicity as; "Toxicity is the adverse end product of a series of events that is initiated by exposure to chemical, physical or biological agents. Toxicity can manifest itself in a wide array of forms, from mild biochemical malfunctions to serious organ damage and death. These events, any of which may be reversible or irreversible, include absorption, transport, metabolism to more or less toxic metabolites, excretion, interaction with cellular macromolecules and other modes of toxic action." (Society of Toxicology, 2003).

Some materials may be toxic on the nanoscale whereas their bulk form may be harmless. For instance, gold in bulk form is inert, however, gold nanoparticles are in exact opposite of bulk form which makes them appropriate for medical imaging and drug delivery applications. Despite that, according to Nature Nanotechnology (2011), "Nanoparticles are also more likely to react with cells and various biological components such as proteins, and to travel through organisms, which increases their chances of entering various organs and activating inflammatory and immunological reponses.".

Biocompatibility of nanoparticles is one of the most important causes of nanoparticle toxicity (Piotrowska, 2009). Kirchner et al. (2005) categorizes nanoparticle toxicity into three main causes as following;

1) Chemical toxicity when Cd^{2+} is released from nanoparticles of cadmium selenide. High area to volume ratio characteristic of nanoparticles causes partial release of ions.

2) Nanoparticles may hold on cellular membranes and get into the cells. Cellular functions may be damaged because of inert nanoparticles that do not decompose or do not react with other matrix components.

3) Shape of the nanoparticle has an impact on toxicity of the particle. For instance, carbon nanotubes can easily penetrate into cell membrane.

2.4.1 Impacts of nanoparticles on human

Skin contact, injection, inhalation and ingestion are the ways of taking nanoparticles to human body. According to Oberdörster (2005), air, water, clothes, drug delivery and food can be nanoparticle sources through human body. These nanoparticles can be found in lymph, bone marrow, kidney, spleen, heart, liver in human body. They are transported by neurons, respirotary tract, blood and gastrointestinal tract, and finally can be seen in sweat, urine, breast milk and feces as seen in following Figure 2. 5;

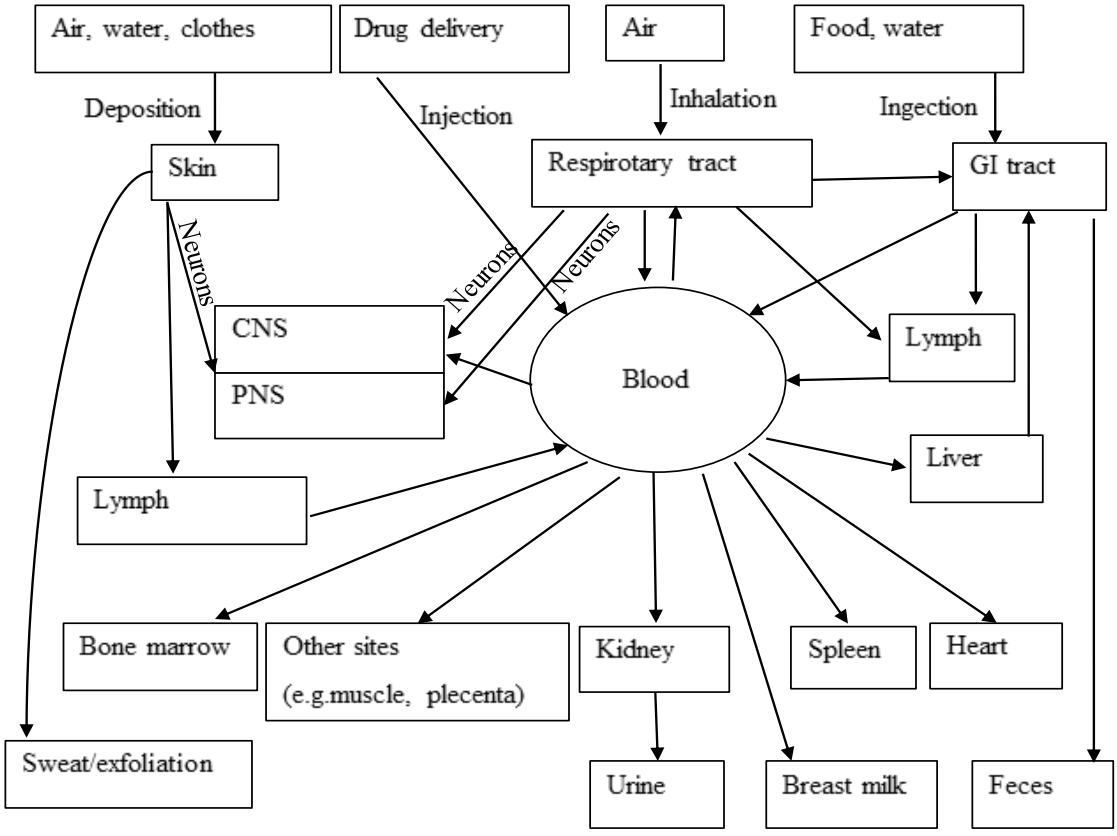


Figure 2. 5 : The pathway of nanoparticles in human body (Oberdörster, 2005).

According to Oberdörster (2008), most of the toxicity research for mammalian systems on nanoparticles are focused on respirotarial uptake to test the significant

health effects of ultrafine particles. According to Oberdörster (2010), the characteristics, biokinetics and effects of nanoparticles versus the effects of larger particles where respirotary tract as portal of entry are summarized in Table 2. 3.

Table 2. 3 : The characteristics, biokinetics and effects of nanoparticles versus larger particles where respirotary tract as portal of entry (Oberdörster, 2010).

General characteristics	**Nanoparticles (>100 nm)**	**Larger particles (<500 nm)**
Ratio:number/surface area per volume	High	Low
Agglomeration in air, liquids	Likely (dependent on medium: surface)	Less likely
Deposition in respirotary tract	Diffusion: throughout resp. tract	Sedimentation, impaction, interception; throughout resp. tract
Protein/lipid adsorption in vitro	Yes; important for biokinetics	Less effective
Translocation to secondary target organs		
Clearance	Yes	Generally not (to liver under overload)
Muccociliary	Probably yes	Efficient
Alv. macrophages	Poor	Efficient
Epithelial cells	Yes	Mainly under overload
Lymphatic circulation	Yes	Under overload
Blood circulation	Yes	Under overload
Sensory neurons (uptake + transport)	Yes	No
Protein/lipid adsorption in vivo	Yes	Some
Cell entry/uptake	Yes (caveolae; clathrin; lip. Rats; diffusion)	Primarily phagocytic cells
Mitochondria	Yes	No
Nucleus	Yes (<40nm)	No

Table 2. 3 (continued): The characteristics, biokinetics and effects of nanoparticles versus larger particles where respirotary tract as portal of entry.

General characteristics	Nanoparticles (>100 nm)	Larger particles (<500 nm)
Direct effects (caveat: chemistry and dose)		
At secondary target organs	Yes	No
At portal of entry (resp. Tract)	Yes	Yes
Inflammation	Yes	Yes
Oxidative stress	Yes	Yes
Activation of signalling pathways	Yes	Yes
Primary genotoxicity	Some	No
Carcinogenity	Yes	Yes

2.4.2 Environmental impacts of nanoparticles

In the environment, there are two different sources of nanoparticles; natural sources such as forest fires and volcanoes, and anthropogenic sources which a group of unintentional sources such as power plants, and intentional sources such as nanoproducts and nano-included products.

The fate of nanoparticles in ecosystem consisting of soil, water and air is depicted in Figure 2. 6. Nanoparticles can be deliberated or accindentally released into one or more environmental components. They may affect photochemical or chemical change, alter surface characteristics or bind to other particles/surfaces and then further dispersal is seen by volatilisation to air, or by leaching to water, or by drainage, surface adsorption to soil which causes persistance or environmental contamination. After that, biological organisms may uptake them which may invlude sources of human food. Consequently, bioaccumulation in food chain, or adverse effects such as ecotoxicological hazard, or biodegradation may be seen (Khan & Arif, 2012).

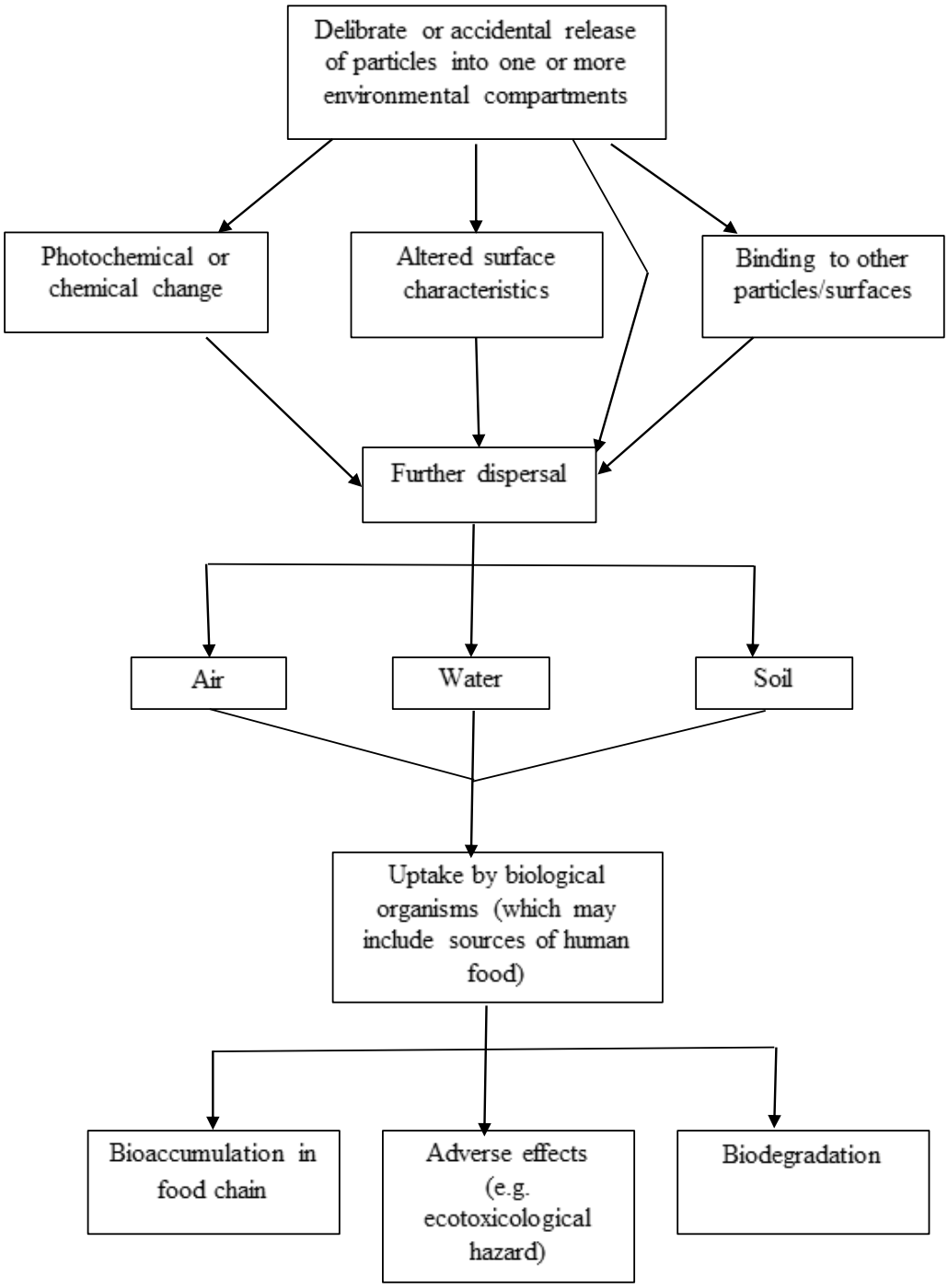


Figure 2. 6 : The fate of nanoparticles in the ecosystem (Khan & Arif, 2012).

2.4.3 Impacts of nanoparticles on microorganisms

In aquatic systems, bacteria and viruses as microbial contaminants in colloids or colloidal mixture forms with particle sizes in micrometer or nanometer scales may be found. Nanosized colloids may have negative impacts on aquatic environment and water quality. Interconnection between nanoparticles and microorganisms, and

effects on growth of nanoparticles on bacteria and viruses are not well known yet. AgNP and ZnONP are used as antimicrobial agents commonly. Particle size, shape, concentration and aggregation/dissolution are the main factors which effect antibacterial activities. According to You et al. (2011), "The antibacterial mechanisms of metallic or metallic oxide nanoparticles include alternation of cell membrane properties and permeability leading to particle penetration inside the bacterial cell and the release of metal ions and DNA damage". Additionally, according to You et al. (2011), "For metallic oxide nanoparticles such as ZnO and TiO_2 NPs, the generation of reactive oxygen species (ROS) via photocatalysis may be responsible for the antimicrobial actions" (You et al., 2011).

Studies of Choi et al. (2008) shows that AgNPs can penetrate into the cell membrane because of their small sizes and can accumulate in the cell which causes problems in the cell. However, some researchers have suggested that AgNPs are hold on cell membrane which causes cell death by affecting permeability of the membrane (Lok et al., 2006; Morones et al., 2005; Sondi and Salopek-Sondi, 2004) Additionally, some other studies shows that cell membranes are weaken by AgNPs due to their damaging impact on enzyme (Morones et al., 2005; Matsumura et al., 2003). Dimkpa et al. (2011) found that AgNPs affects electron transport without affecting cell membrane. AgNPs affect the growth of the cells, correspondingly antibacterial mechanism by damaging the cell DNA, while there is a contribution of AgNPs by Ag^+ on antibacterial mechanism (Sun et al. (2013). According to Sun et al. (2013), "The overall negative charge of bacterial extracellular polymeric substances (EPS) repels AgNPs which are also negatively charged and thus protects bacteria from AgNPs toxicity. EPS can also quench reactive oxygen species (ROS) generated by AgNPs treatments or EPS may bind Ag^+ released from AgNPs and reduce the extent of cellular contact. However, EPS protection can be circumvented at high concentrations of AgNPs.".

2.4.4 Impacts of nanoparticles on wastewater treatment processes

Activated sludge processes are the most used processes for municipal wastewater treatment plants. Activated sludge includes heterotrophic bacteria and autotrophic nitrifying bacteria. Its activity is important for the removal of BOD and nutrients,. Toxic compounds affect the activity of these bacterias (Liu et al., 2011). However, there are not enough research reports on the fate of engineered nanoparticles during

wastewater treatment processes which are important for accurate environmental risk assessments of nanomaterials (Hou et al., 2012).

Unavoidably, engineered nanoparticles are released into natural environment primarily from sewage and industrial discharges due to the increase in the production and application of those nanoparticles. For instance, AgNPs contained nanotextiles release their nanoparticle content during washing (Hou et al., 2012).

According to the study of Rickard Arvidsson at Gothenburg's waste water treatment plant in Sweden, amount of silver used in clothings has a direct effect on sludge, and agricultural land if sludge is used as fertilizer. In the same study, Arvidsson mentions the silver concentration in the examined clothing varied by a factor of one million -- between 0.003 mg/kg and 1400 mg/kg. There would not be any observable effect with the lowest concentration, otherwise Rickard Arvidsson says, "With the highest concentration, however, it would suffice if all of the city's residents bought and used one silver nanoparticle-treated sock a year for the silver concentration in waste water treatment plant sludge to double" (Arvidsson, 2012)

Removal of nanoparticles are related with biosolids which are removed by sedimentation and/or filtration. When biosolids are applied to land, terrestial organisms are exposed to nanoparticles. Also, aquatic organisms are exposed when nanomaterials are not removed form wastewater treatment plant. They may discharge in the water into rivers, lakes, and oceans (Wang et al., 2012). Usage scenarios for emission of different products comprising nanomaterials into wastewater treatment plants are given in Table 2. 4. Nanoparticle contents of those products are literally not available.

Table 2. 4 : Usage scenarios for emission of different products comprising nanomaterials into wastewater treatment plants (Brar et al., 2010).

Product Type	**Emission (g/pc/d)**
Antiperspirant	0.35
Body lotion	1.2
Body was	0.32
Cleaners	0.3
Deodorants	0.08
Face cream	1.64
Hair conditioner	0.47

Table 2. 4 (continued): Usage scenarios for emission of different products comprising nanomaterials into wastewater treatment plants

Product Type	Emission (g/pc/d)
Hair styling products	0.10
Lime deposit removers	0.11
Paint	0.09-0.36 ml/pc/yr
Laundry detergants	10.1-20.5
Oral hygiene products	0.7
Perfumes	0.05
Shampoo	1.83-6.30
Shaving foam	0.07
Soap	2.5
Skin care products	1.3
Softeners	16.4
Sunscreen	3.0
Window cleaners	0.03

3. EXPERIMENTAL METHOD

3.1 Materials

Silver (Ag^0, <100 nm) and aluminium (Al^0, 25 nm) nanoparticles were purchased from NanoAmor (Texas, USA). Synthetic domestic wastewater was prepared by using glucose, urea, $(NH_4)_2SO_4$, $NaHCO_3$, $MgSO_4$, KH_2PO_4 (Merck), K_2HPO_4 (Merck) (Table 3. 1). Formaldehyde (37%) and NaOH used for biological substances analysis were purchased from Merck. For protein analysis, folin, Na_2CO_3, Na_2tartarate.$2H_2O$, BSA and $CuSO_4.5H_2O$ were bought from Merck and for carbohydrate analysis phenol and H_2SO_4 (%95-97) were also purchased from Merck. No further purification were applied to the chemicals used in this study.

Table 3. 1 : The composition of the synthetic domestic wastewater.

Chemicals	**Amount (mg/L)**
Glucose	1000
Urea	100
KH_2PO_4	50
K_2HPO_4	50
$(NH_4)_2SO_4$	50
$MgSO_4.7H_2O$	50
$NaHCO_3$	250

3.2 Preparation of the Membrane Solutions

3.2.1 Mixed liquor suspended solids (MLSS)

Before feeding the aerated activated sludge, 5 mL activated sludge sample was taken from the experimental medium on daily basis. A filter was taken from a dessicator and its tare was weighted (m_1). Activated sludge sample was filtered from MLSS set under vacuum conditions. The filter was dried in oven at 105°C for one hour. Upon

drying, the filter was waited in the desiccator for 1 h., and then the filter was weighed (m_2). MLSS is calculated by following equation;

$$\text{MLSS}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{(m_2 - m_1)}{\text{ml sample volume}} \text{x}1000 \quad \textbf{(3.1)}$$

3.2.2 Biological substances analysis (extracellular polymeric substances-EPS)

Extracellular polymeric substances (EPS) are the construction materials for microbial aggregates such as biofilms, flocs and activated sludge liquors. The term "EPS" is used as a general and comprehensive concept for different classes of macromolecules such as polysaccharides, proteins, nucleic acids, (phosphor-)lipids and other polymeric compounds which have been found at or outside the cell surface and in the intercellular space of microbial aggregates. They consist of insoluble materials (sheaths, capsular polymers, condensed gel, loosely bound polymers and attached organic material) produced by active secretion, shedding of cell surface material or cell lysis. The term "EPS" is used as a general parameter to characterize bio-polymeric substances in the reactor. It is important to recognize that the exact definitions of eEPS (extracted extracellular polymeric substances), and sEPS (soluble extracellular polymeric substances) are directly dependent of the methods used to obtain and characterize chemically these solutions. In literature, it has been now widely accepted that the concepts of sEPS and soluble microbial products (SMP) are identical. Also only EPS term was generally used for eEPS parameter (Le-Clech, et. al., 2006). The usage of EPS terms is given at Figure 3. 1.

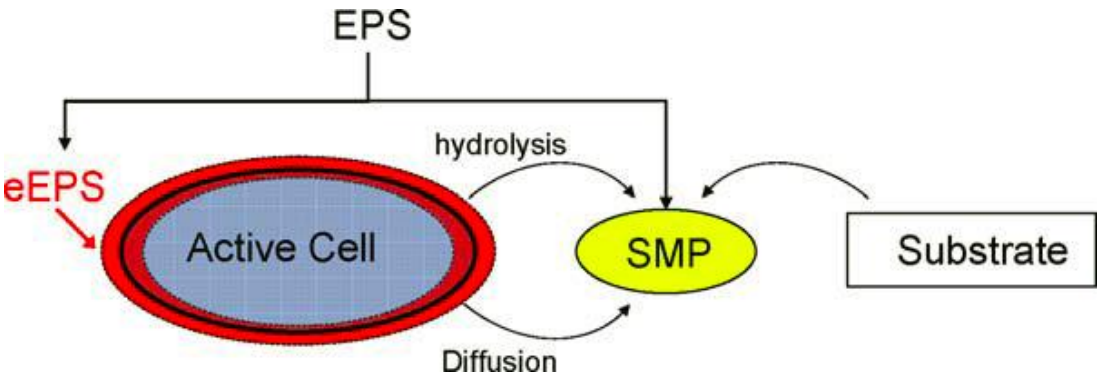


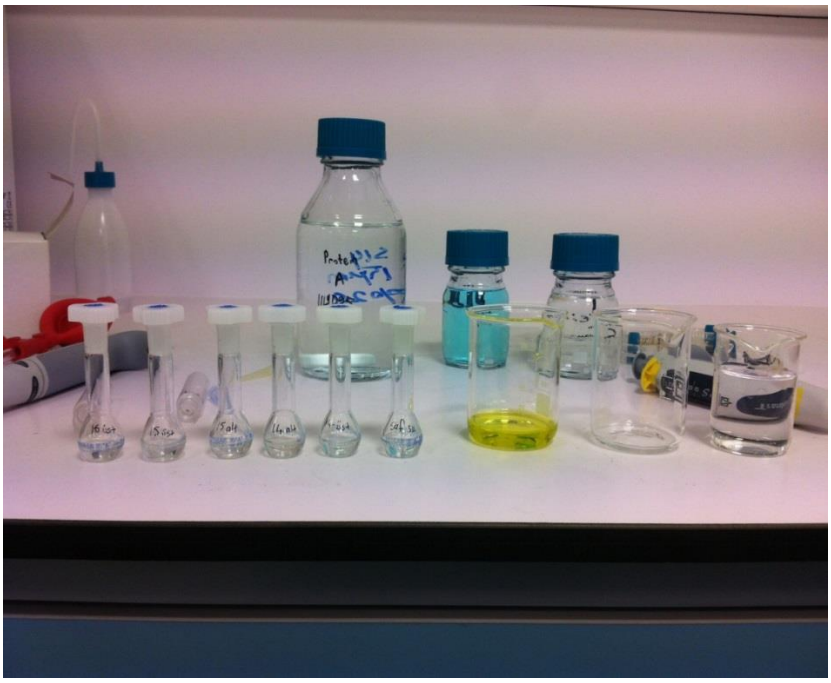
Figure 3. 1 : Simplified representation of EPS, eEPS and SMP (Le-Clech, et. al., 2006).

In this study, SMP and EPS analysis were carried out on daily samples of activated sludge. For those analysis, physical-chemical (sodium hydroxide – formaldehide) extraction method was used. Formaldehide prevents cellular decomposition by reacting with nucleic acids, and amino, hidroxile, carboxil and sulfide groups of proteins in cell membrane. Consequently, cellular forms are protected. NaOH increases the pH, and the pH of the solution of EPS is increased, hence, more EPS is extracted from the cell. 5 mL of activated sludge from each experimental vessel was taken into a centrifuged tube and then the suspension was centrifuged (4000×g, 10min, 4^0C). The supernatant was decanted into another sterile tube and recentrifuged (13.200×g, 20min, 4^0C) to ensure complete removal of the suspended solids. The resultant supernatant by this physical extraction contained soluble carbohydrate and soluble protein, and was analyzed for soluble EPS content (SMP). The solid flocs in the tube were resuspended with distilled water to obtain another 5mL suspension. Then, 6 µL formaldehyde (37%) was added first into the suspension for 1 h at 4 ◦C, followed by 0.5 mL NaOH (1N) for another 3 h at 4 ◦C. The suspension was centrifuged (13.200×g, 20min, 4 ◦C) and the supernatant from this chemical extracting method, containing bound carbohydrate and bound protein, was analyzed for bound EPS content. Carbohydrate concentrations were quantified by the phenol–sulfuric acid method. Protein concentrations were determined by using the modified Lowry method. Carbohydrate and protein analysis methods are given in following sections.

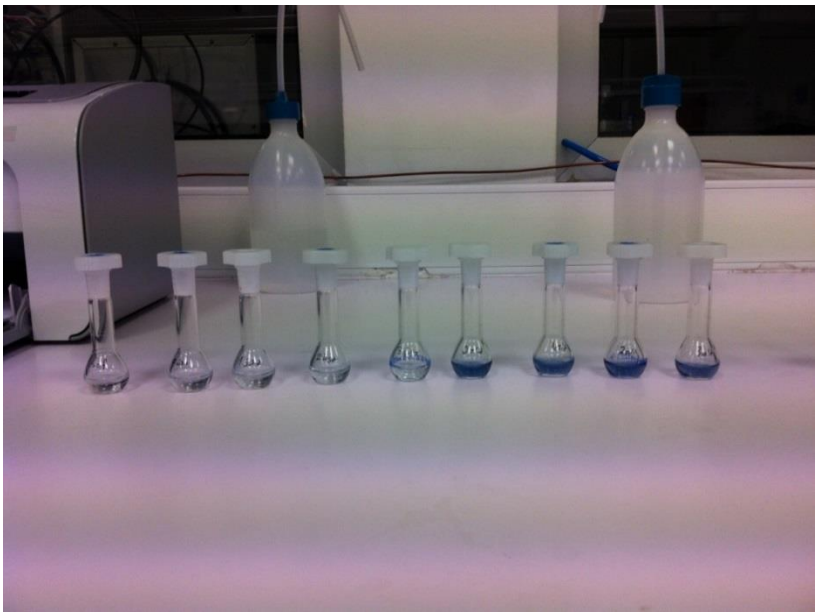
3.2.3 Protein analysis method

The real-time photographs of protein analysis in the experimental system are given at Figure 3. 2. Three main solutions (A,B and C solutions) were prepared for analysis of Lowry protein method. 2.86 g NaOH and 14.31 g Na_2CO_3 were dissolved in distilled water and diluted to 500 ml for preparation of the solution A. 1.42 g $CuSO_4.5H_2O$ was dissolved in 100 ml distilled water for preparation of the solution B. And, 2.85g Na_2tartarate.$2H_2O$ was dissolved in 100 ml distilled water for preparation of the solution C. Lowry solution was prepared by mixing these three solutions with a specific rate of 100:1:1 (A:B:C) right before the analysis. 0.7 ml Lowry solution was added to 0.5 ml sample and stirred for 20 min at room temperature in a dark place. Folin solution was prepared during this stage. Then, 5 ml of 2N Folin solution was mixed with 6 ml of distilled water. 0.1 ml of folin solution was mixed with 0.5 ml of

the sample and kept in a dark place for 30 min after vigorous stirring. Afterwards, samples were colored from light to dark blue according to their protein concentrations. Measurements were done by using a UV spectrophotometer, obtained from Hach Lange Company, at a constant wavelength (660 nm). Reference solution was also prepared under the same conditions for the measurements. Two similar solutions were prepared and measured for each solution in order to provide repeatability for the measurements.



(a)



(b)

Figure 3. 2 : The photographs of protein analysis experimental systems.

Bovin Serum Albumin (BSA) was used as the standard protein solution for protein calibration. Solutions having concentrations in the range of 0-100 mg/L were prepared by using the standard protein. Absorption values were measured at 660 nm by using a UV spectrophotometer. The absorption-concentration graph was drawn with the obtained values. Calibration graph is given in the Figure 3. 3.

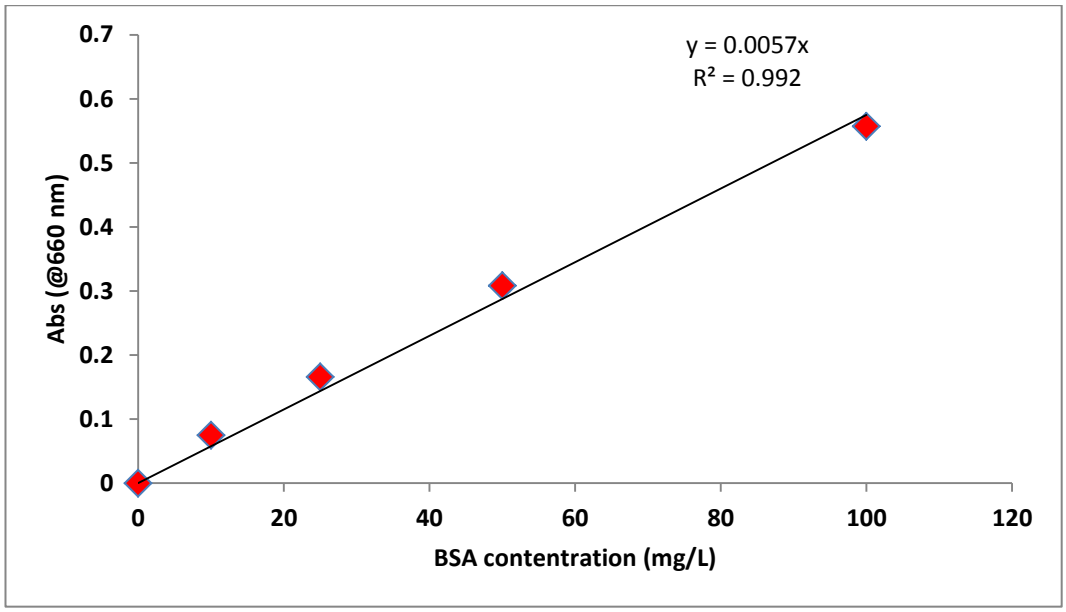


Figure 3. 3 : Calibration curve for protein analysis.

3.2.4 Carbohydrate analysis method

Modified "phenol-sulfuric acid method" was used for the carbohydrate analysis. Phenol solution with concentration of 80% and concentrated H_2SO_4 (95-97%) were used for the analysis. 25 µL of phenol and 2.5 mL of H_2SO_4 were added to 1 mL of sample at 30^0C and kept in a water bath for 15 min. Colors of the samples were varied from light yellow to dark yellow according to their carbohydrate concentrations. Colors were investigated by using a UV spectrophotometer at 490 nm wave length. The photograph of carbohydrate analysis is given at Figure 3. 4. Glucose was used as the standard for calibration. Solutions having concentrations in the range of 2-80 mg/L were prepared by using the standard glucose. Calibration curve is shown in the Figure 3. 5.

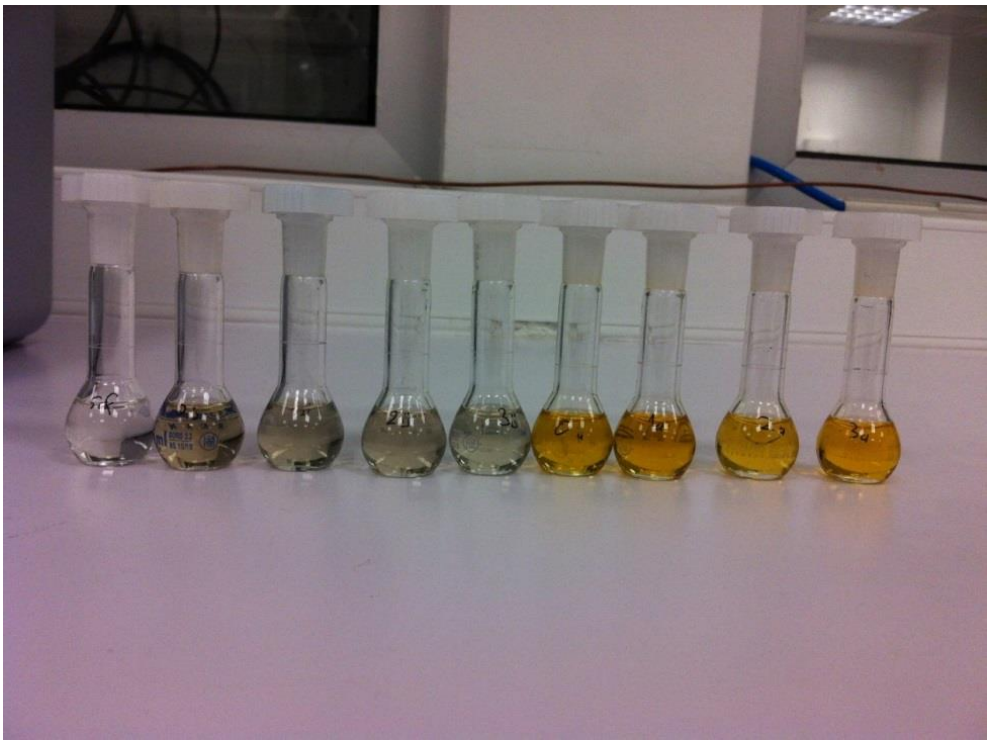


Figure 3. 4 : The photograph of carbohydrate analysis experimental systems.

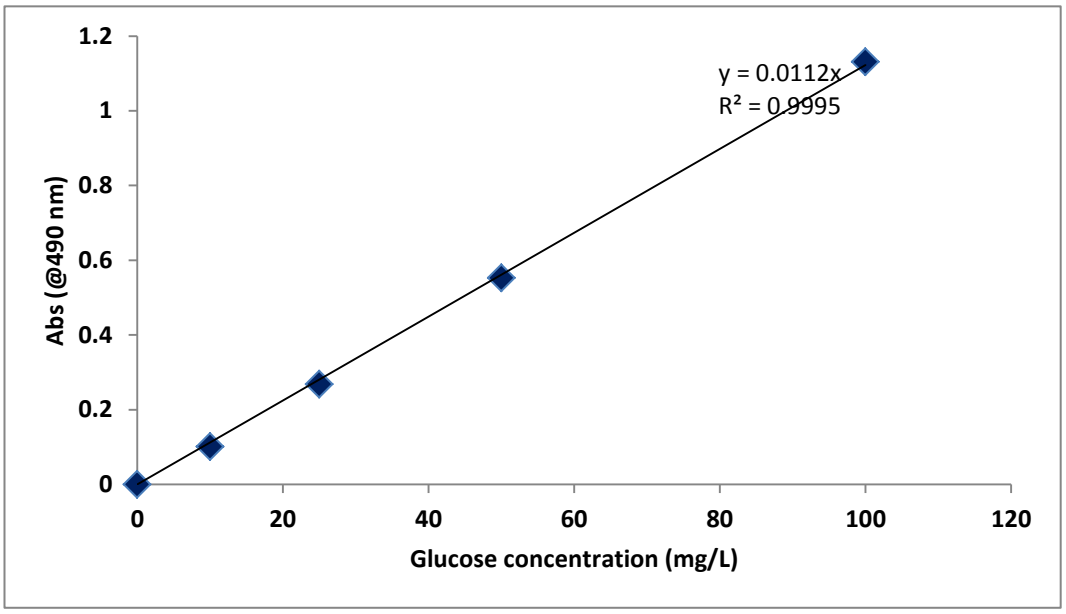


Figure 3. 5 : Calibration curve for carbohydrate analysis.

3.2.5 Optical density

During long term analysis, in order to observe the bacterial growth and the setttling properties at activated sludge, the optical density measurement was carried out both at activated sludge and at supernatant of activated sludge after settling time of 30 min. For this analysis, 3 mL of samples were taken from activated sludge and

supernatant suspensions. Then, OD (optical density) values were scanned in Hach-Lange DB5000 spectroscopy device at a wavelength of 600 nm. OD values measured at activated sludge and supernatant were named as OD_{sludge} and $OD_{supernatant}$.

3.2.6 Viscosity measurement

During long term analysis, viscosity values of the activated sludge were measured by using an AND - Vibro Viscometer-SV10 (measures the viscosity by using wave lengths). Viscosity measurements were carried out at room temperature by using approximately 40 ml of activated sludge. Images of the device and the measurements are shown in Figure 3. 6. Distilled water's viscosity was always measured at first place and the device was calibrated according to measured value, and after that samples were analysed.

Figure 3. 6 : Image of the viscosimetry measurement process and the viscosimeter.

3.2.7 Inductively coupled plasma (ICP) analysis

The total silver and aluminium concentrations in the samples were analyzed using ICP device. Total concentrations of them were quantified using a Perkin-Elmer (Norwalk, CT) Optima 3000 DV Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). All measurements were carried out in the axial mode. A 999 ±0.2 µg/ml standard solution from Inorganic Ventures including silver and

aluminum, water and %5 HNO3 (v/v) was used as an internal standard for calibration as recommended by the ICP manufacturer and correlation coefficient was 0.9998. The detection limits of silver and aluminum for the ICP was 0.01 mg/L. Before the analysis, the samples were filtered with using a syringe filter (0.45 µm pore size).

3.3 Experimental Setup

Experiments were carried out under four main title. Those are;

- Dissolution test: the determination of dissolution of the nanoparticles in pure water at different experimental conditions,
- Short-term nanotoxicity test: High concentrations, that are selected due to previous experiment, of nanoparticle load into activated sludge in short term experiment,
- Long-term nanotoxicity test: Low concentrations of nanoparticle load into activated sludge in long term experiment,
- Respirometric toxicity test: the determination of respiration rate with dissolved oxygen measurements to calculate toxicity values of nanoparticles.

Procedures of experiments are given in the following titles.

3.3.1 Dissolution tests

In the dissolution experiments, the effects of pH, temperature, stirring rate, and NP ratio on dissolution of AlNP and AgNP were examined. Experimental matrix is given in Table 3. 2 . In the first experiment, three different pH values (3.0, 5.0 and 7.0) were tested while the temperature, stirring rate and NP ratio were constant. In the second experiment, three different temperature values (25.0, 35.0 and 45^{o}C) were tested in optimum pH value while the mixture rate and NP ratio are constant. In the third experiment, four different mixture rate values (200, 400, 600 and 800 rpm) were tested in optimum pH and temperature values while NP ratio are constant. In the last experiment, four different NP ratio values (0.1, 0.2, 0.6 and 1%) were tested under optimum pH, temperature, stirring rate and NP ratio conditions. ICP analysis was used for determination of dissolution ratios of NPs. The dissolution ratio of NPs was calculated as shown in Eq.3.2;

$$\text{Solubility ratio (\%)} = 1 - \frac{C_t}{C_s} \quad \textbf{(3.2)}$$

$C_{t:}$ The calculated initial concentration which was added to solution (mg/L).

C_s: The measured concentration in solution by ICP device (mg/L).

Table 3. 2 : The experimental matrix of dissolution test

Run	Parameters	Values	Experimental conditions			
			pH	T (^{o}C)	Rate (rpm)	NP ratio (%)
1	pH	3 5 7	-	25	200	0.4
2	T (^{o}C)	25 35 45	7.0	-	200	0.4
3	Stirring rate (rpm)	200 400 600 800	7.0	25	-	0.4
4	NP ratio (%, m/m)	0.1 0.2 0.6 1.0	7.0	25	400	-

3.3.2 Short-term nanotoxicity test

Effects of AgNP and AlNP on biological properties of activated sludge were firstly carried out with short-term tests. The preparation and testing protocol of AgNP and AlNP were completely identical and thus NPs symbol is used for both nanoparticles in following sentences. The optimum conditions according to the dissolution experiments were selected for these experiments. Activated sludge samples were freshly taken from aeration tank of Kocaeli Tertiary Biological Treatment Plant. In these experiments, three different NPs ratios (0.05-0.1-0.2%, m/m) were studied, while other experimental conditions were constant. For the preparation of stock solutions containing NPs, firstly NPs were added to the distilled water and dissolved completely by using a sonication probe for 60 min. After that, the nanomaterials

were dissolved completely, the stock solutions were continued to dissolve by stirring for 24 h. After the NPs were completely dissolved, these stock solutions were added to the synthetic domestic wastewater and stirred again with a magnetic stirrer for 1 h in order to obtain homogeneous solution. The photograph of NPs stock solutions is given at Figure 3. 7. These mixture containing both wastewater and NPs was fed to the activated sludge samples. Before feeding, the samples were taken for anlaysis at the beginning of the experiment. Blank activated sludge was fed with domestic wastewater without NPs. After the feeding of activated sludge with mixture, these activated sludge and blank activated sludge were kept in a shaker at a constant stirring rate during experiments. Experiments were durated as 3 hour and the samples were taken from vessels for SMP and EPS analysis at every hour.

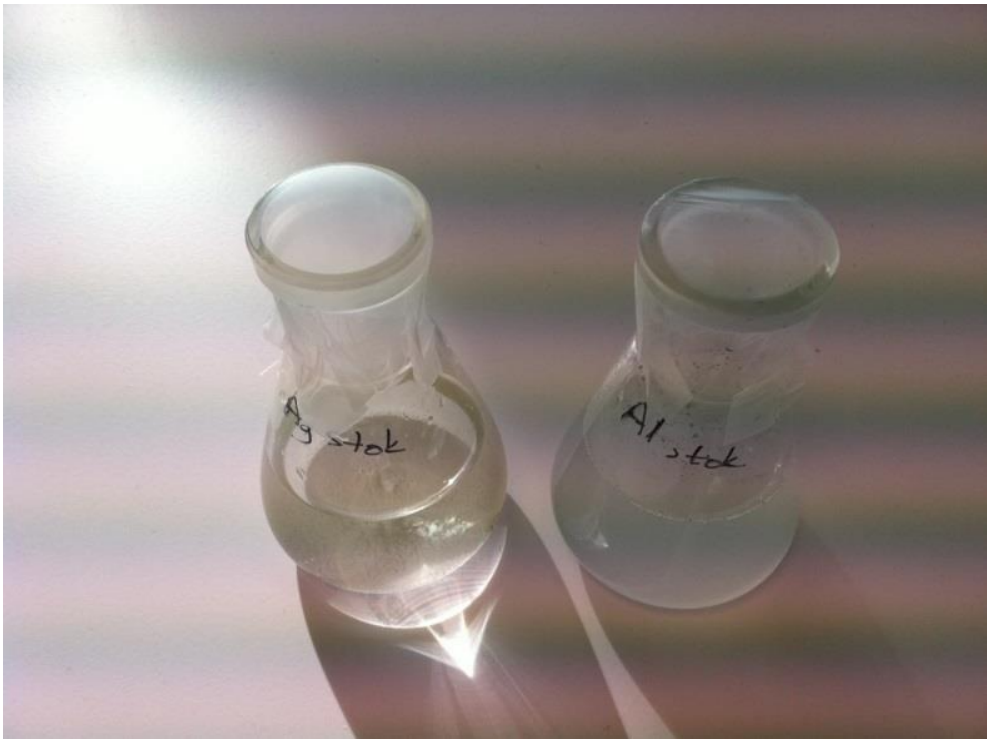


Figure 3. 7 : The photograph of NPs stock solutions

3.3.3 Long-term nanotoxicity test

In this section, the preparation and long-terms testing protocol of AgNP and AlNP were completely similar and thus NPs symbol is used for both nanoparticles in following sentences. In this part of study after short-term nanotoxicity analysis, the total concentration of NPs (used at short term tests) were gradually added to the activated sludges in 10 days period. Thus, the determination of cumulative effects of NPs in long term at activated sludge were investigated. Similarly to the short-term tests, the activated sludge samples were freshly taken from aeration tank of Kocaeli

Tertiary Biological Treatment Plant. Differently from the short term tests, the diffusers having constant aeration rate were used, in this way, the real activated sludge conditions were simulated in laboratory scale. In the experiments, the activated sludge suspension having same biological parameters were equally separated as one blank sample (without NP) and three samples having different NP ratios. The activated sludge samples were only aerated without feeding in the first 24 hours to remove the residual organics. After that, the first NPs ratios (0.005-0.01-0.02%) and synthetic domestic wastewater were added to these activated sludge samples and then the experiments were launched. The feeding of NPs and synthetic domestic wastewater was carried out daily (at same time) in same activated sludge samples so that the cumulative NP ratios reached to 0.05-0.1-0.2% at the end of 10 days experiment. The blank activated sludge was fed with only domestic wastewater. Synthetic domestic wastewater was prepared according to Table 3. 1 at daily, NPs were added to this solution and sonicated for 1 h. Experimental schema is shown in the following Figure 3. 8.

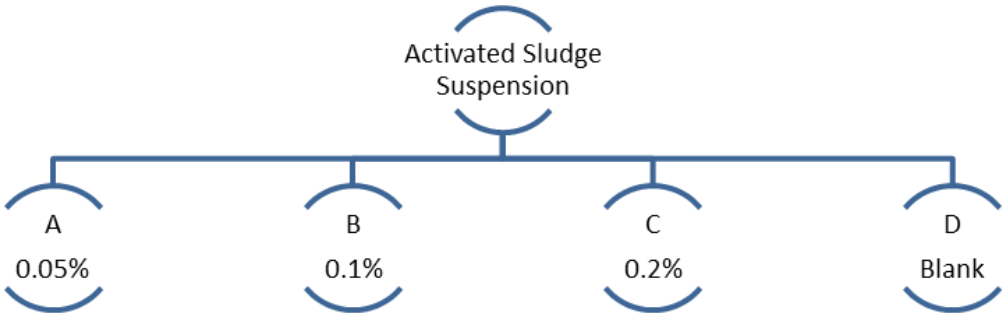


Figure 3. 8 : The experimental shematic diagram of long term nanotoxicity test

Biological parameters (MLSS, OD_{sludge}, $OD_{supernatant}$, viscosity, SMP and EPS) of activated sludge suspension were analysed daily during the long term experiments. At the same time, ICP measurements were done daily to determine dissolved concentration of NPs in sludge. 0.45 µm syringe filters were used to infiltrate sludge samples before ICP analysis. The real-time photograph of long-term experiments is given at Figure 3. 9.

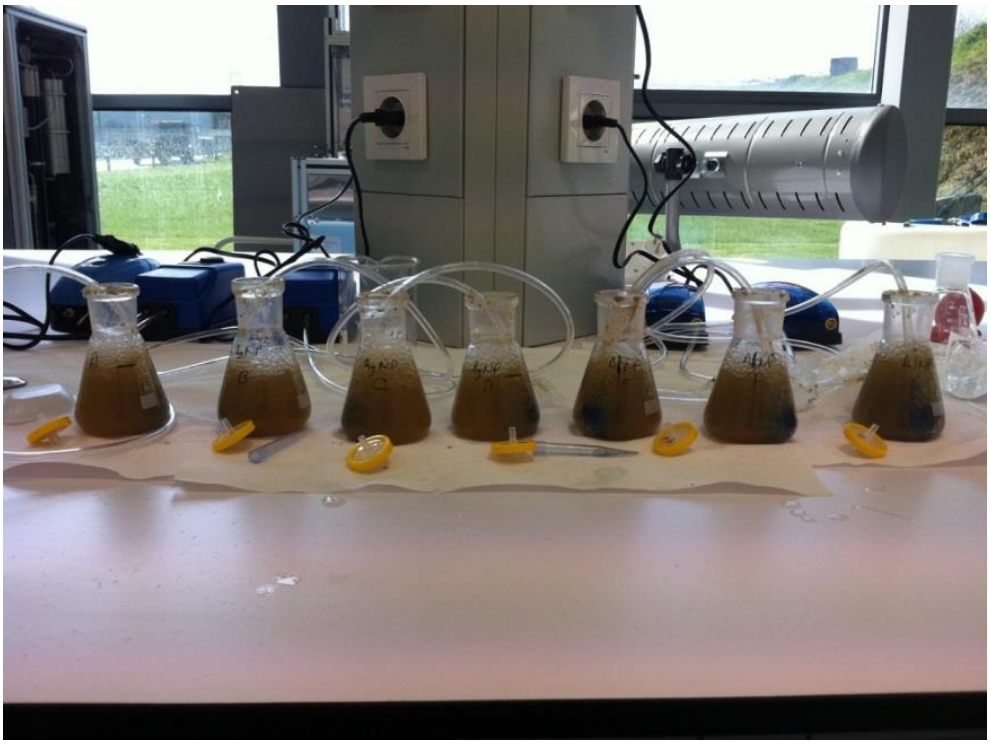


Figure 3. 9 : The real-time photograph of long-term experiments

3.3.4 Respirometric toxicity test

Bacterial growth inferred from oxygen uptake rates due to NPs toxicity was measured in triplicate using a batch extant respirometric test. The activated sludge samples were collected from the lab-scale activated sludge reactor. The activated sludge bacterial suspensions were amended with AgNPs and AlNPs individually at the final concentration range of 0.05-0.1-0.2%. For each respirometric test, 50 mL of activated sludge, with an average concentration of mixed liquor suspended solids (MLSS) of 2400 mg/L, was aerated and stirred overnight to ensure that all the substrate present in the sludge was consumed. Respiration tests were performed at 25^0C. Aeration was then stopped. The dissolved oxygen (DO) decrease, without external substrate addition, was measured for 10 min by using an oxygen meter (Hach-Lange) connected to a PC. This procedure was repeated three times and the average of the slope of the DO decrease was taken as endogenous oxygen uptake rate (OUR_{end} in mg O_2/g MLSS.h). Afterwards, the aeration was reinitiated and a pulse (10 mg/mL) of readily biodegradable chemical oxygen demand in the form of glucose was added. This procedure was repeated three times to calculate the average OUR. The exogenous OUR (OUR_{ex}) was obtained by subtracting the previously determined OUR_{end} from the OUR value obtained from the available substrate.

Finally, the biological suspension was left to settle for 1 h and the upper portion (~10 mL) was removed and substituted with the corresponding nanoparticle suspension. The loss of biomass and specific biological activity during this procedure was negligible when it is measured by overall respiration. The whole experimental procedure was repeated in the presence of AgNP and AlNP nanoparticles to obtain the OUR_{NP}. The inhibition percentage was calculated as the OUR was reduced, with and without nanoparticles, and after 3 h exposure time. The experimental stages of respirometric toxicity test is given in Figure 3. 10.

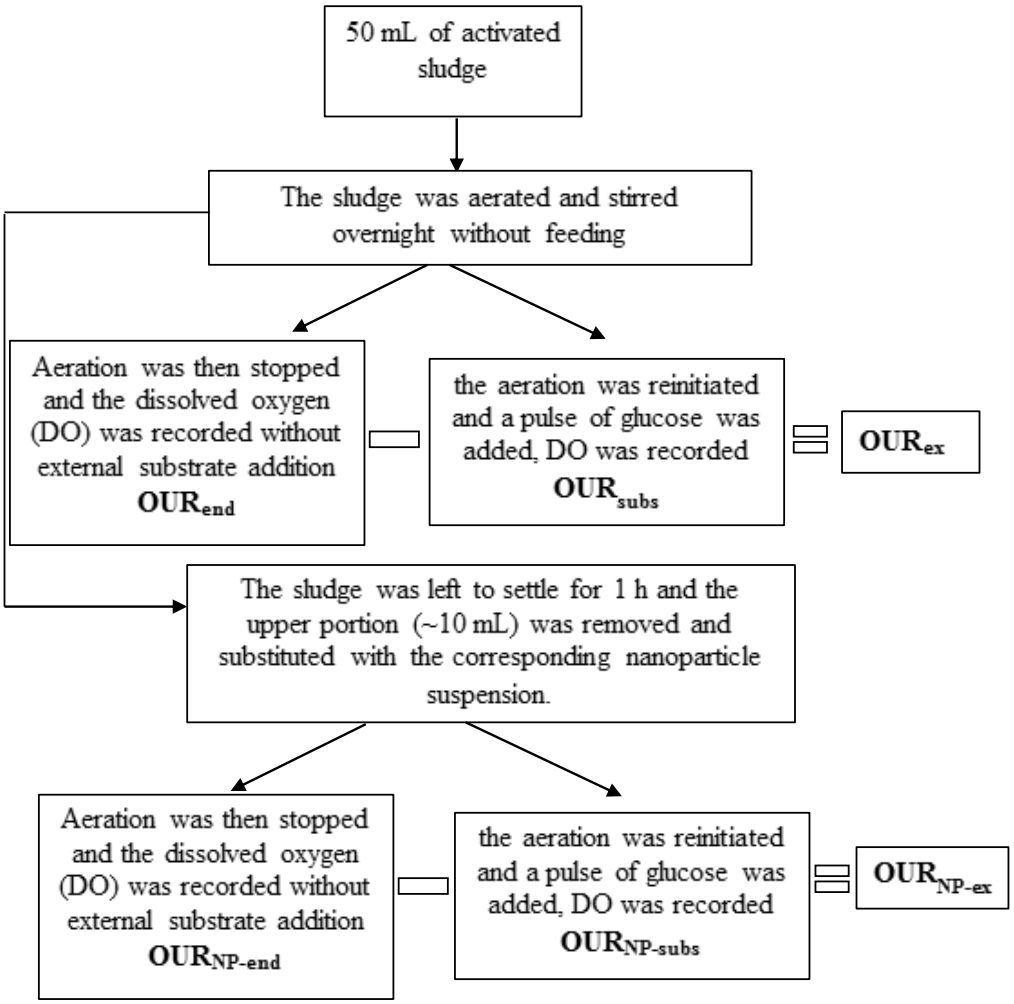


Figure 3. 10 : The experimental stages of respirometric toxicity test

$$Inhibition\ ratio(\%) = \left(1 - \frac{OUR_{NP-ex}}{OUR_{ex}}\right) x100 \qquad \textbf{(3.3)}$$

4. EXPERIMENTAL RESULTS

4.1 Experimental Results of Dissolution Analysis

Experimental results of dissolution analysis are given particularly for AgNP and AlNP as following.

4.1.1 The dissolution performance of AgNPs

The dissolution performance of AgNP results are given for pH, temperature, stirring rate and AgNP ratio as following.

4.1.2 The effect of pH on dissolution of AgNP

In the pH experiments, three different pH values (3.0, 5.0 and 7.0) were tested while the temperature (at 25^0C), stirring rate (at 200 rpm) and NP ratio (at 0.4%, m/m) were constant. The pH value of the samples were measured as 6.96 (~7.0) and acidic solution is used for pH adjustment. The dissolution ratio of AgNPs was calculated using Eq.(3.2) given in section 3.3.1. The graph of dissolution ratios is given at Figure 4. 1 and the values were calculated as 9.6, 6.3 and 12.0% for pH 3.0, 5.0 and 7.0, respectively. ICP device measures the ionic silver (Ag^+). These results shows that the dissolution of AgNP was low in all pH values but it can be said that the maximum dissolution was observed at neutral pH (pH 7.0) whilst the minimum dissolution was observed at pH 5.0. According to that analysis, the optimum pH was determined as pH 7.0.

4.1.3 The effect of temperature on dissolution of AgNP

In the temperature experiments, three different temperature values (25.0, 35.0 and 45.0^0C) were tested while the pH (at 7.0), stirring rate (at 200 rpm) and NP ratio (at 0.4%, m/m) were constant. The temperature experiments were carried out at a termostatically controlled water bath. The dissolution ratio of AgNP was calculated using Eq.(3.2) given in section 3.3.1. The graph of dissolution ratios is given in

Figure 4. 2 and the values of dissolution ratios were calculated as 14.6, 13.2 and 12.0% for 25^0C, 35^0C and 45^0C, respectively. The dissolution of AgNP was decreased with increasing temperature. However, it can be said that the maximum dissolution was observed at room temperature (T=25^0C). As a result, the optimum temperature was determined as 25^0C.

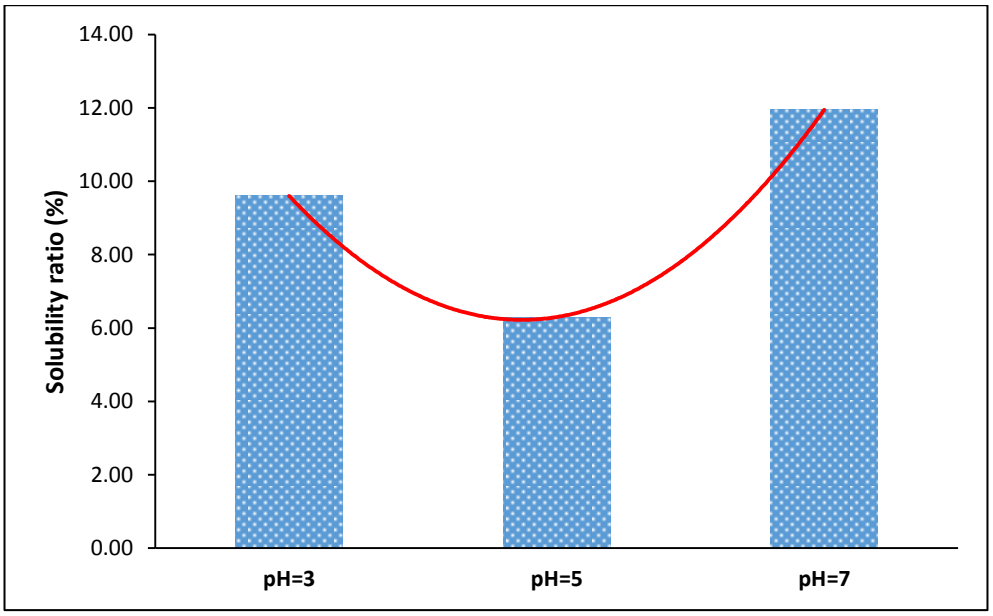


Figure 4. 1 : Dissolution ratio of AgNPs at different pH values.

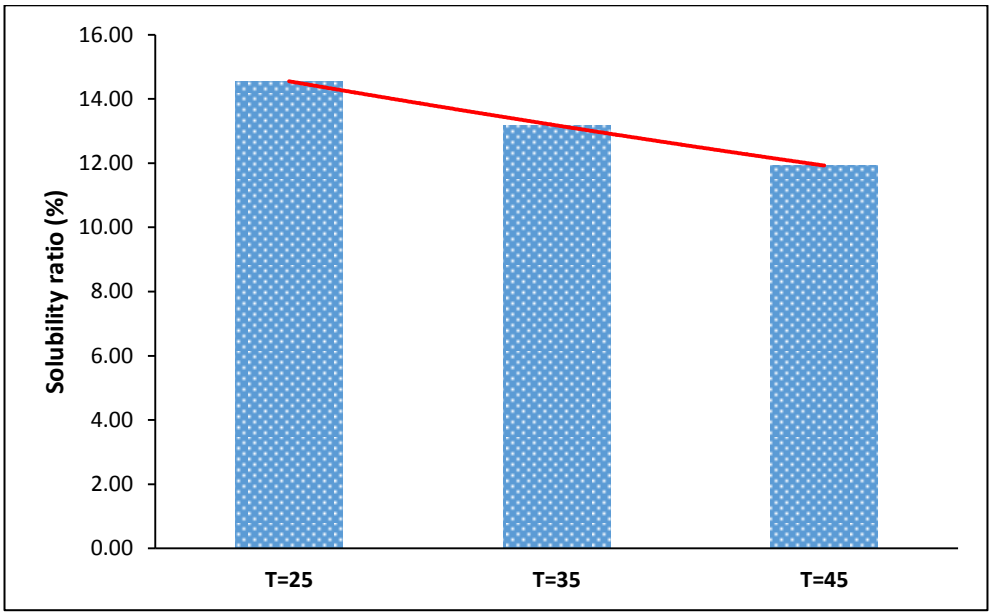


Figure 4. 2 : Dissolution ratio of AgNPs at different temperature values.

4.1.4 The effect of stirring rate on dissolution of AgNP

In the striring rate experiments, four different stirring rate values (200, 400, 600 and 800 rpm) were tested while the pH (at 7.0), temperature (at 25^{0}C) and NP ratio (at 0.4%, m/m) were constant. The stirring rate experiments were carried out at rotational shaker. The dissolution ratio of AgNP was calculated using Eq.(3.2) given at section 3.3.1. The graph of dissolution ratios is given in Figure 4. 3 and the values of dissolution ratios were calculated as 14.7, 27.6, 12.5 and 5.5% for 200, 400, 600 and 800 rpm, respectively. The dissolution of AgNPs was increased with increasing stirring rate from 200 rpm to 400 rpm but then it decreased with increasing stirring rate. Therefore, it can be said that the maximum dissolution was observed at stirring rate of 400 rpm. As a result, the optimum stirring rate was determined as 400 rpm.

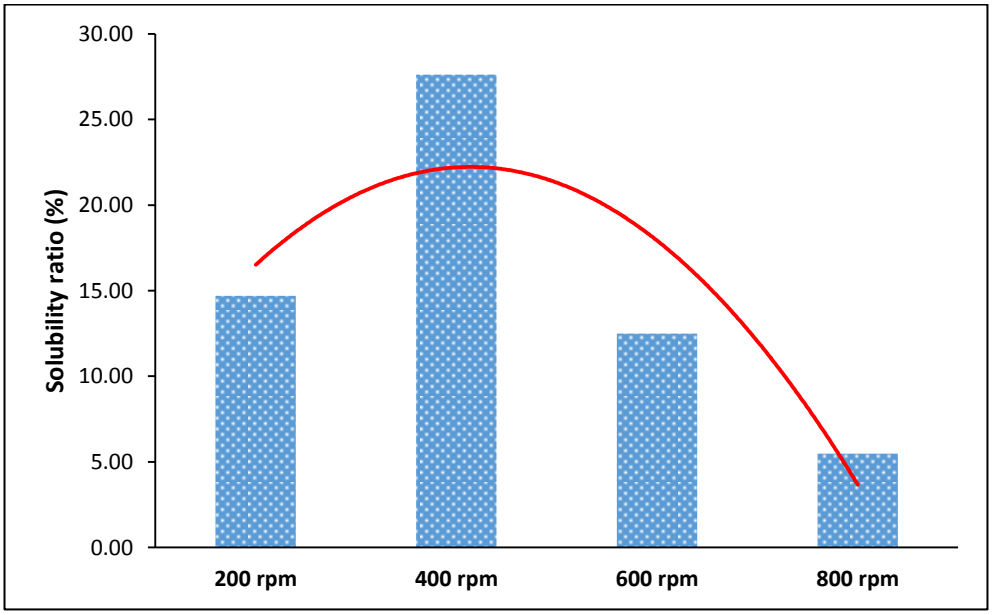


Figure 4. 3 : Dissolution ratio of AgNPs at different stirring rate values.

4.1.5 The effect of AgNPs ratios on dissolution of AgNP

In the AgNPs ratio experiments, four different AgNPs ratio values (0.1, 0.2, 0.6 and 1.0%) were tested while the pH (at 7.0), temperature (at 25^{0}C) and stirring rate (at 400 rpm) were constant. The AgNP ratio experiments were carried out at rotational shaker. The dissolution ratio of AgNP was calculated using Eq.(3.2) given at section 3.3.1. The graph of dissolution ratios is given in Figure 4. 4 and the values of dissolution ratios were calculated as 70.7, 47.8, 17.2 and 14.5% for 0.1, 0.2, 0.6 and 1.0%, respectively. The dissolution of AgNPs was dramatically decreased with increasing AgNPs ratio. It can be concluded that, the increasing concentration of

AgNPs ratio may cause agglemoration of nanoparticles in solution. So the dissolution efficiency was declined. Therefore, it can be said that the maximum dissolution was observed at 0.1% AgNP ratio. As a result, the optimum AgNP ratio was determined as 0.1%.

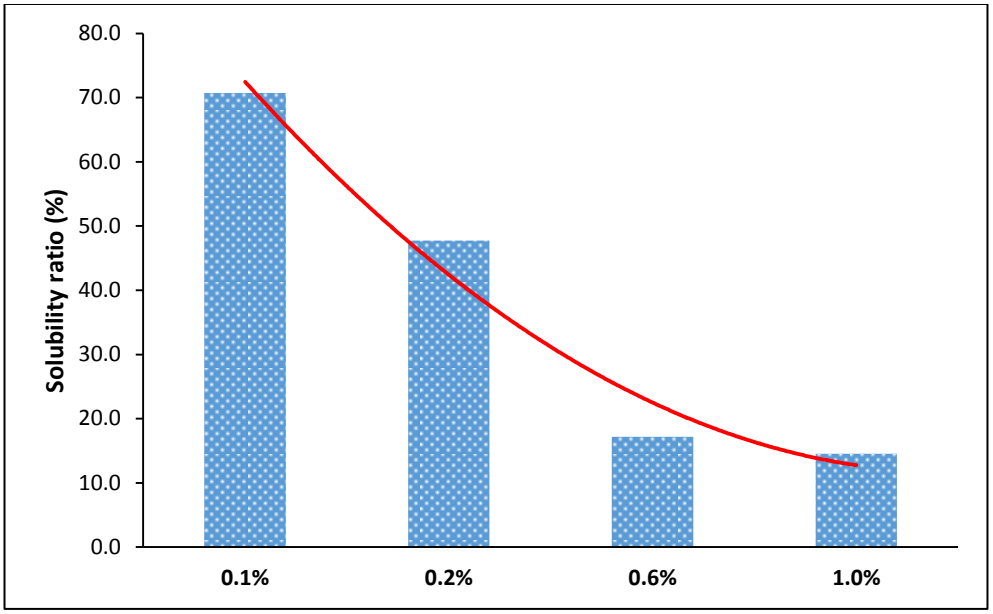


Figure 4. 4 : Dissolution ratio of AgNPs at different AgNPs amounts.

From the results of AgNP dissolution tests, the optimum conditions were determined as pH=7.0, T=25^{0}C, stirring rate=400 rpm and the ratio of AgNP=0.1%. For this reason, these values were used at short-term, long-term and respirometric toxicity tests.

4.1.6 The dissolution performance of AlNPs

The dissolution performance of AlNP results are given for pH, temperature, stirring rate and AlNP ratio as following.

4.1.7 The effect of pH on dissolution of AlNP

In the pH experiments, three different pH values (3.0, 5.0 and 7.0) were tested while the temperature (at 25^{0}C), stirring rate (at 200 rpm) and NP ratio (at 0.4%, m/m) were constant. The pH value of the samples were measured as 6.3 and acidic solution is used for pH adjustment. The dissolution ratio of AlNPs was calculated using Eq.(3.2) given at section 3.3.1. The graph of dissolution ratios is given in Figure 4. 5 and the values were calculated as 36.3, 32.2 and 32.2% for pH 3.0, 5.0 and 7.0, respectively. These results showed that the dissolution of AlNPs was higher than the dissolution of AgNPs at different pH. It can be said that, the maximum

dissolution was observed at acidic pH (pH 3.0) but the change of dissolution was not very remarkable. The acidic pH can damage the bacterial cells, thus, it was decided that the real pH value of AlNPs solution was optimal for toxicity tests.

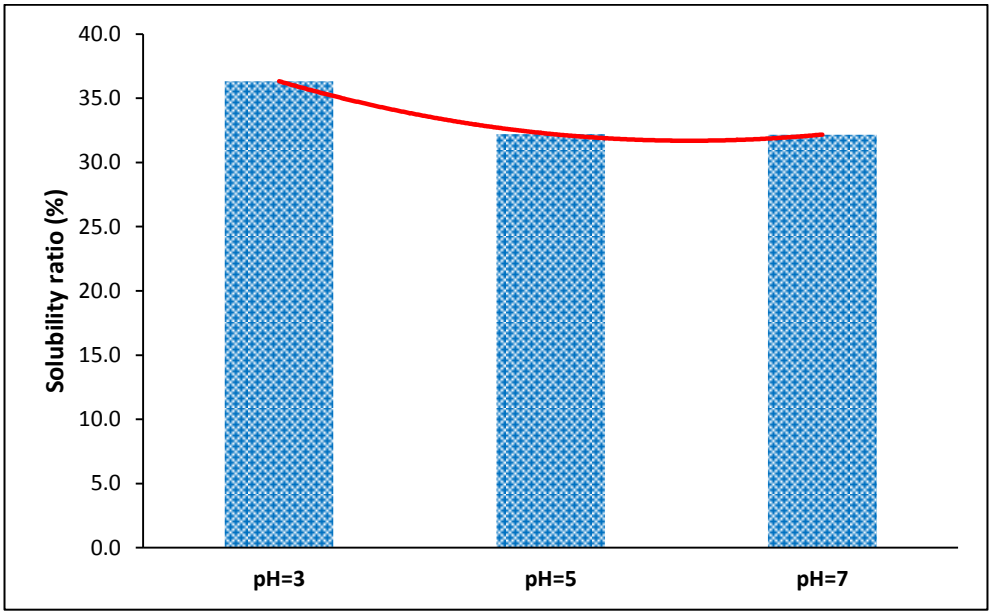


Figure 4. 5 : Dissolution ratio of AlNPs at different pH values.

4.1.8 The effect of temperature on dissolution of AlNP

In the temperature experiments, three different temperature values (25.0, 35.0 and 45.0^{0}C) were tested while the pH (at 7), stirring rate (at 200 rpm) and NP ratio (at 0.4%, m/m) were constant. The temperature experiments were carried out at termostatically controlled water bath. The dissolution ratio of AlNPs was calculated using Eq.(3.2) given at section 3.3.1. The graphs of dissolution ratios are given in Figure 4. 6 and the values of dissolution ratios were calculated as 35.1, 32.6 and 33.7% for 25^{0}C, 35^{0}C and 45^{0}C, respectively. The dissolution of AlNPs did not change with increasing temperature. However, it can be said that the optimum temperature was determined as room temperature (25^{0}C).

4.1.9 The effect of stirring rate on dissolution of AlNP

In the striring rate experiments, four different stirring rate values (200, 400, 600 and 800 rpm) were tested while the pH (at 7), temperature (at 25^{0}C) and NP ratio (at 0.4%, m/m) were constant. The stirring rate experiments were carried out at rotational shaker. The dissolution ratio of AlNP was calculated using Eq.(3.2) given at section 3.3.1. The graphs of dissolution ratios is given in Figure 4. 7 and the values

of dissolution ratios were calculated as 43.1, 32.2, 32.9 and 33.4% for 200, 400, 600 and 800 rpm, respectively. The dissolution of AlNPs decreased with increasing stirring rate from 200 rpm to 400 rpm. However, no effect was observed for stirring rates higher than 400 rpm. Therefore, it can be said that the maximum dissolution was observed at stirring rate of 200 rpm. As a result, the optimum stirring rate was determined as 200 rpm.

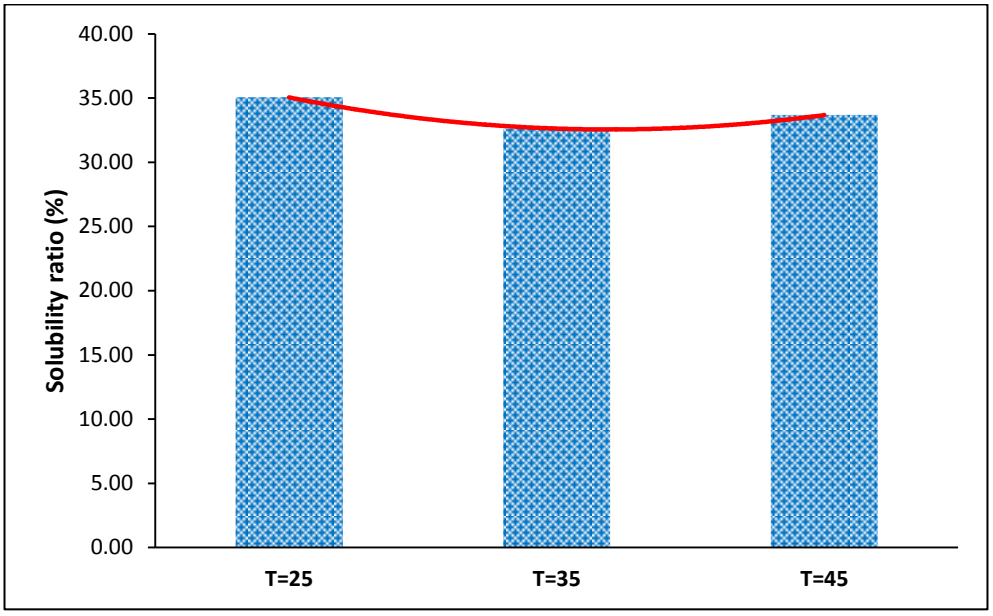


Figure 4. 6 : Dissolution ratio of AlNPs at different temperature values.

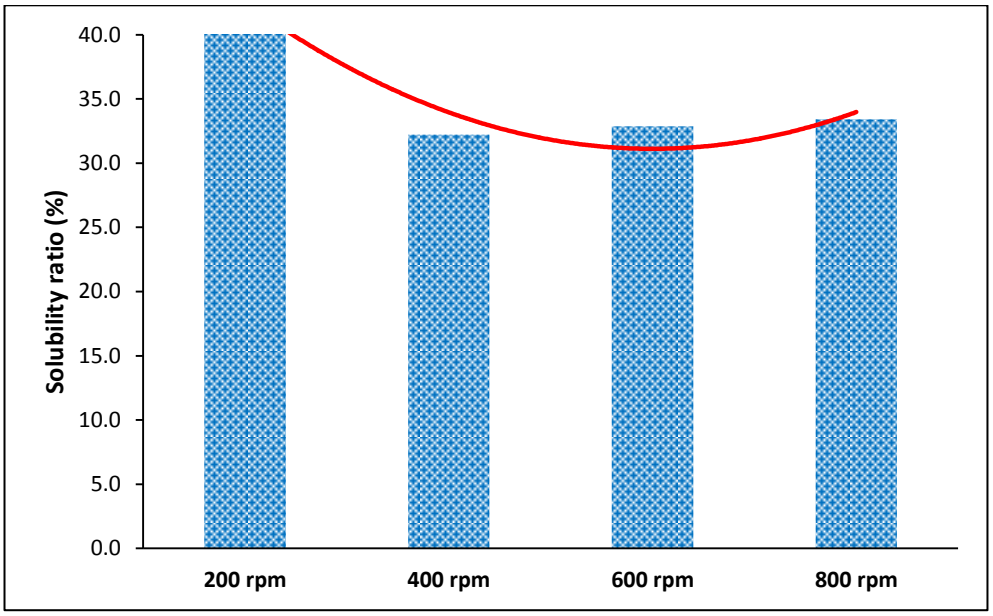


Figure 4. 7 : Dissolution ratio of AlNPs at different stirring rate values.

4.1.10 The effect of AlNPs ratios on dissolution of AlNP

In the AlNPs ratio experiments, four different AlNPs ratio values (0.1, 0.2, 0.6 and 1.0%) were tested while the pH (at 7), temperature (at 25^0C) and stirring rate (at 200 rpm) were constant. The AlNPs ratio experiments were carried out at rotational shaker. The dissolution ratio of AlNPs was calculated using Eq.(3.2) given at section 3.3.1. The graph of dissolution ratios is given in Figure 4. 8 and the values of dissolution ratios were calculated as 82.0, 66.7, 23.0 and 14.0% for 0.1, 0.2, 0.6 and 1.0%, respectively. Similar to AgNPs experiments, the dissolution of AlNPs was dramatically decreased with increasing AlNPs ratio. It can be concluded that, the increasing concentration of AlNPs ratio may cause the agglemoration of nanoparticles in solution. So the dissolution efficiency declined. Therefore, it can be said that the maximum dissolution was observed at 0.1% AlNP ratio. As a result, the optimum AlNP ratio was determined as 0.1%.

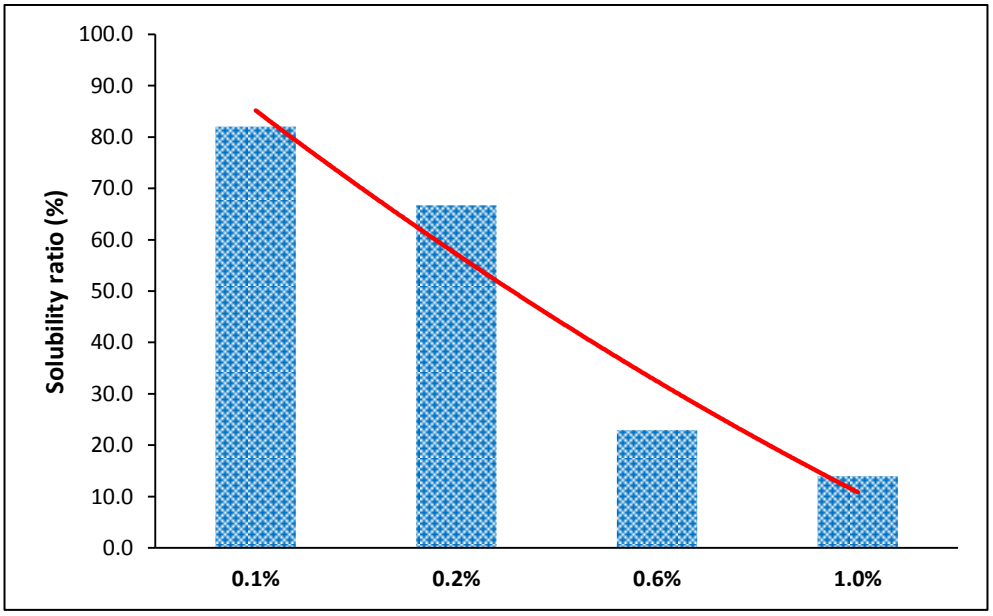


Figure 4. 8 : Dissolution ratio of AlNPs at different AlNPs amounts.

From the results of AlNP dissolution tests, the optimum conditions were determined as pH=6.3, T=25^0C, stirring rate=200 rpm and the ratio of AlNP=0.1%. For this reason, these values were used at short-term, long-term and respirometric toxicity tests.

4.2 Results of Short-term Nanotoxicity Tests

Results of short-term nanotoxicity tests are given particularly for AgNP and AlNP as following.

4.2.1 The effect of AgNPs on activated sludge properties

Effects of AgNPs on biological properties of activated sludge were firstly carried out with short-term tests. The optimum experimental conditions were determined according to dissolution experiments as pH=7.0, temperature=25.0^{0}C, stirring rate=400 rpm and three different NPs ratios (0.05-0.1-0.2%, m/m). In the short-term tests, SMP and EPS analysis were carried out at every hour during 3 h experiment. The MLSS concentration of activated sludge samples were averagely found as 2267±76 mg/L. The graphs of SMP analysis are given in Figure 4. 9(a, b) according to the protein (SMP_p) and carbonhydrate (SMP_c) contents.

As it can be seen from Figure 4.9(a) that, the addition of 0.05% AgNP to the activated sludge, has no significant effect for SMP_p. However, when 0.1% AgNP was added, an increasing trend was observed during 3-h experiment. When 0.2% AgNP was added to activated sludge, SMP_p maked a peak in the second hour, then decreased back to its initial values. As a result, it may be said that, the effects of AgNPs on SMPp of the activated sludge is related with nanoparticle ratio.

As it can be seen from Figure 4.9(b) that, the addition of 0.05, 0.1 and 0.2% AgNP to activated sludge, has no effect on SMP_c. For 0.1% AgNP, a slight increase was observed at first and third hours but in the second hour it turned to the normal values. The increase was neglected due to their inconsiderable amounts. As a result, it may be said that, the SMPc of the activated sludge is not effected due to AgNPs ratio at the end of 3-h period.

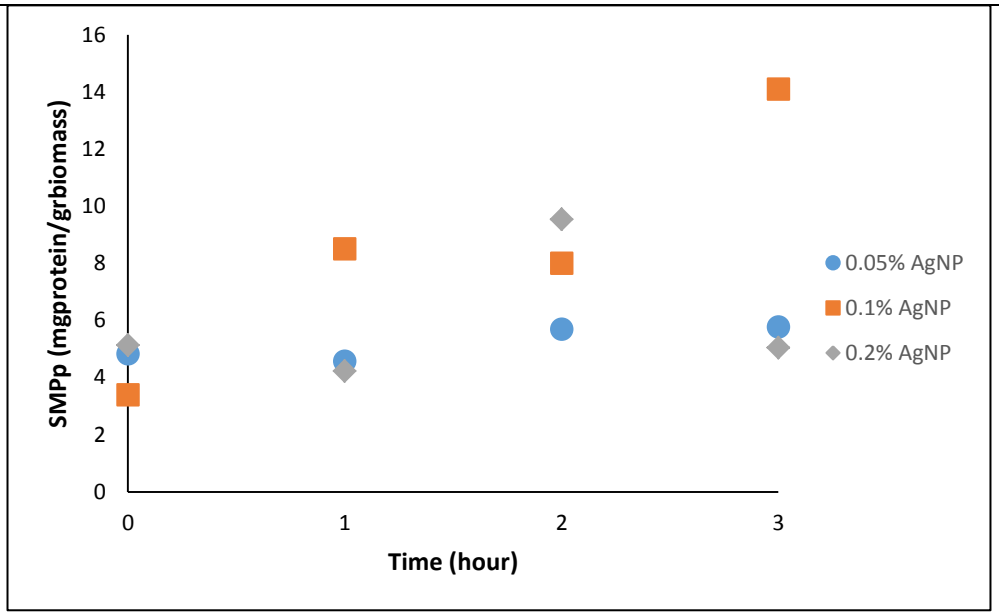
Moreover, the overall effects of AgNPs on SMP content in activated sludge were relatively calculated using SMP values at the beginning (time=0) and at the end of experiment (time=3 h) by Eq (4.1);

$$\text{Overall effects of AgNP} = \left(1 - \frac{\text{SMP}_{t=0}}{SMP_{t=3}}\right) x100 \qquad \textbf{(4.1)}$$

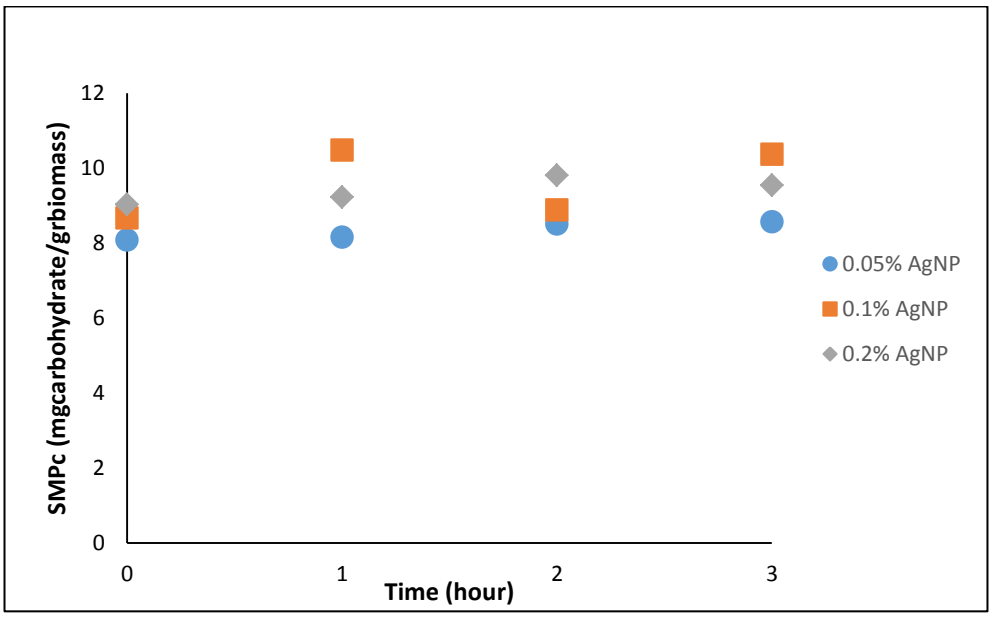
The overall effects of AgNPs on SMP contents are given in Table 4. 1. The signs of (+) and (-) means the increase and decrease, respectively. The highest overall effects were observed at 0.1% ratio of AgNPs and inconsiderable amount of overall changes were observed in both 0.2% AgNPs ratios on SMP contents.

Table 4. 1 : The values of overall effects of AgNPs on SMP contents

	Overall effects (%)	
AgNPs ratios (%)	SMP_p	SMP_c
0.05	(+) 16	(+) 6
0.1	(+) 76	(+) 16
0.2	(-) 2	(+) 5



(a)



(b)

Figure 4. 9 : The graphs of SMP analysis at the short-term nanotoxicity test using AgNPs (a) SMP_p (mgprotein/grbiomass) (b) SMP_c (mgcarbohydrate/grbiomass)

The graphs of EPS analysis are given in Figure 4. 10(a, b) according to the protein (EPS_p) and carbohydrate (EPS_c) contents.

As it can be seen from Figure 4.10(a) that, the addition of 0.05% AgNP to the activated sludge, a significant effect for EPS_p was observed but when 0.1 and 0.2% AgNP were added, a similar trend was observed during 3-h experiment. The concentration of EPSp was the highest at 0.05% AgNP. As a result, it may be said that, the EPSp of the activated sludge is not affected with AgNPs ratio.

As it can be seen from Figure 4.10(b), that the addition of 0.05, 0.1 and 0.2% AgNP to activated sludge, EPS_c concentration increased significantly. The increasing trends of EPS_c were similar at all ratio of AgNPs. As a result, it may be said that, the EPSc of the activated sludge is increased due to increasing AgNPs ratio.

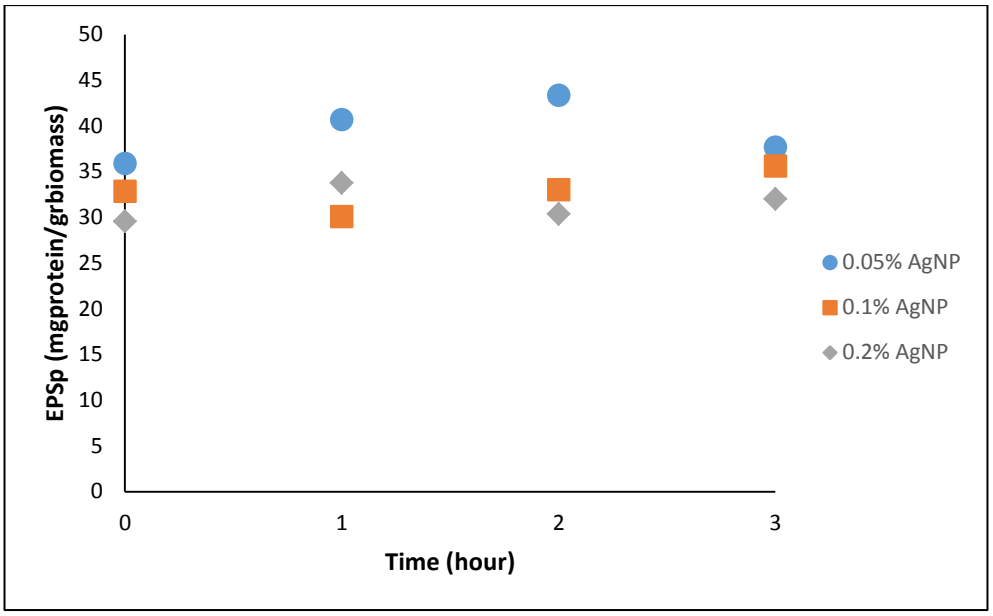
Moreover, the overall effects of AgNPs on EPS content in activated sludge were relatively calculated using EPS values at the beginning (time=0) and end of experiment (time=3 h) following Eq (4.2);

$$\text{Overall effects of AgNP} = \left(1 - \frac{EPS_{t=0}}{EPS_{t=3}}\right) x100 \qquad \textbf{(4.2)}$$

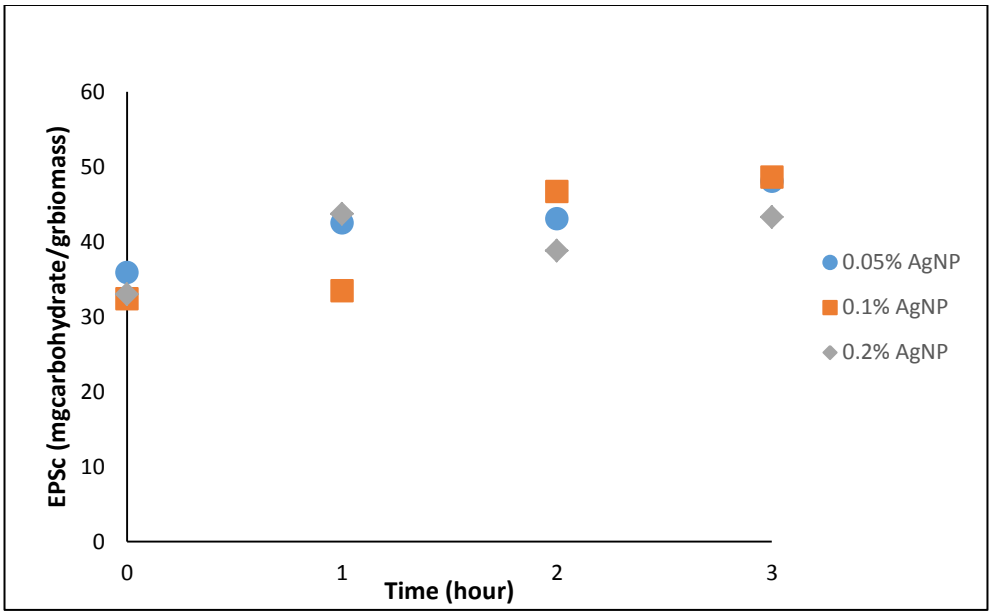
The overall effects of AgNPs on EPS contents are given in Table 4. 2. The signs of (+) and (-) means the increase and decrease, respectively. Non of the AgNPs ratios showed remerkable change on EPS_p. Increasing trends were observed for all AgNPs rations on SPM_c, where the highest overall effects were observed at 0.1% ratio of AgNPs.

Table 4. 2 : The values of overall effects of AgNPs on EPS contents.

	Overall effects (%)	
AgNPs ratios (%)	EPS_p	EPS_c
0.05	(+) 5	(+) 25
0.1	(+) 8	(+) 33
0.2	(-) 8	(+) 24



(a)



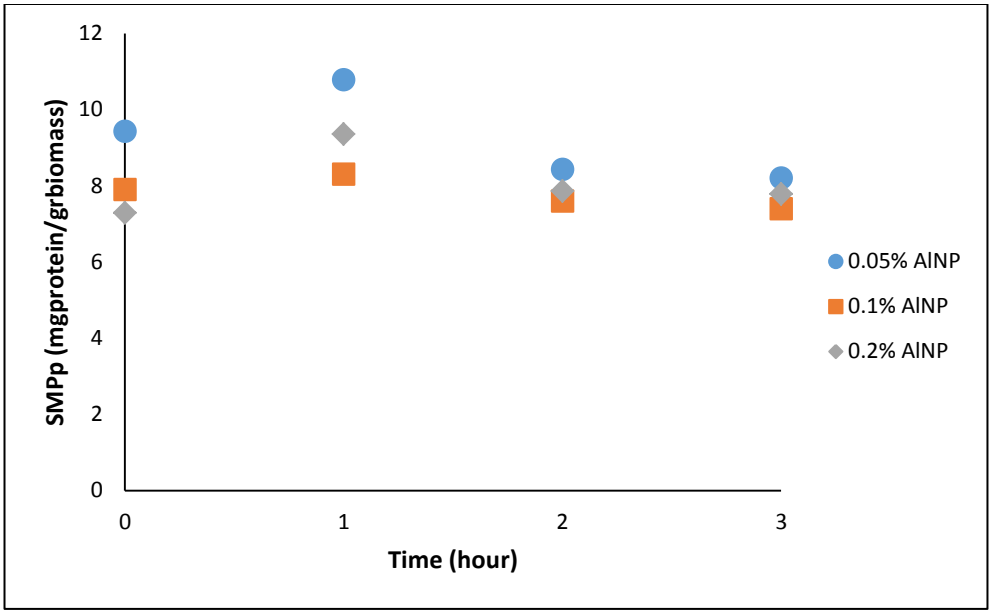
(b)

Figure 4. 10 : The graphs of EPS analysis at the short-term nanotoxicity test using AgNPs (a) EPS_p (mgprotein/grbiomass) (b) EPS_c (mgcarbohydrate/grbiomass)

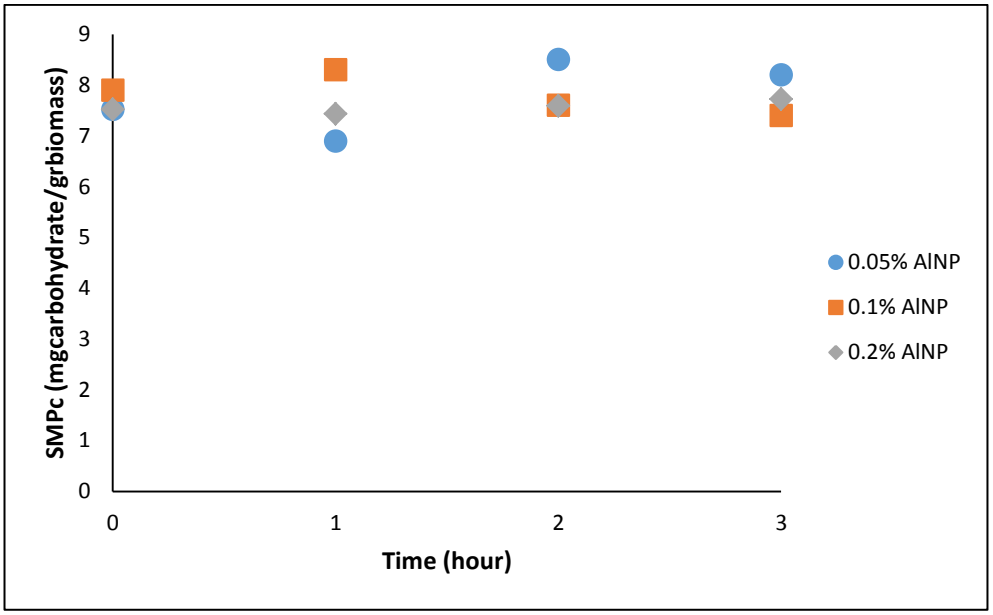
4.2.2 The effect of AlNPs on activated sludge properties

Effects of AlNPs on biological properties of activated sludge were firstly carried out with short-term tests. The optimum conditions according to the dissolution experiments were selected for this experiments as pH=6.3, temperature=25.0^{0}C, stirring rate=200 rpm and three different NPs ratios (0.05-0.1-0.2%, m/m). In this

short-term tests, SMP and EPS analysis were carried out at every hour during 3 h experiment. The MLSS concentration of activated sludge samples were averagely found as 2847±70 mg/L. The graphs of SMP analysis are given in Figure 4. 11(a, b) according to the protein (SMP_p) and carbohydrate (SMP_c) contents.



(a)



(b)

Figure 4. 11 : The graphs of SMP analysis at the short-term nanotoxicity test using AlNPs (a) SMP_p (mgprotein/grbiomass) (b) SMP_c (mgcarbohydrate/grbiomass)

As it can be seen from Figure 4.11(a) that, for all AlNP ratios in the activated sludge, an increasing trend was observed for the first hour but then SMP_p concentrations

were decreased. As a result, it may be said that, the SMPp of the activated sludge is not affected due to AlNPs ratio.

As it can be seen from Figure 4.11(b) that, the addition of 0.05, 0.1 and 0.2% AlNP to activated sludge, has no significant effect on SMP_c which is similar to the short-term tests of AgNPs. As a result, it may be said that, the SMPc of the activated sludge is not affected due to AlNPs ratio.

The overall effects of AlNPs on SMP contents are given in Table 4. 3. The signs of (+) and (-) means the increase and decrease, respectively. The highest overall effect on SMP_p was observed for 0.05% ratio of AlNPs, but was not remarkable to overrate. It can be said that, non of the AlNPs ratios shows remerkable affects on SMP contents.

Table 4. 3 : The values of overall effects of AlNPs on SMP contents

	Overall effects (%)	
AlNPs ratios (%)	SMP_p	SMP_c
0.05	(-) 15	(+) 8
0.1	(-) 7	(-) 7
0.2	(+) 6	(+) 3

The graphs of EPS analysis are given in Figure 4. 12(a, b) according to the protein (EPS_p) and carbohydrate (EPS_c) contents. As it can be seen from Figure 4.12(a) that, in the addition of 0.2% AlNPs to the activated sludge, an increasing affect for EPS_p was observed but when 0.1 and 0.05% AlNPs were added, decreasing trends were observed during 3-h experiment. As a result, it may be said that, the effects of AlNPs on EPSp of the activated sludge is related with nanoparticle ratio.

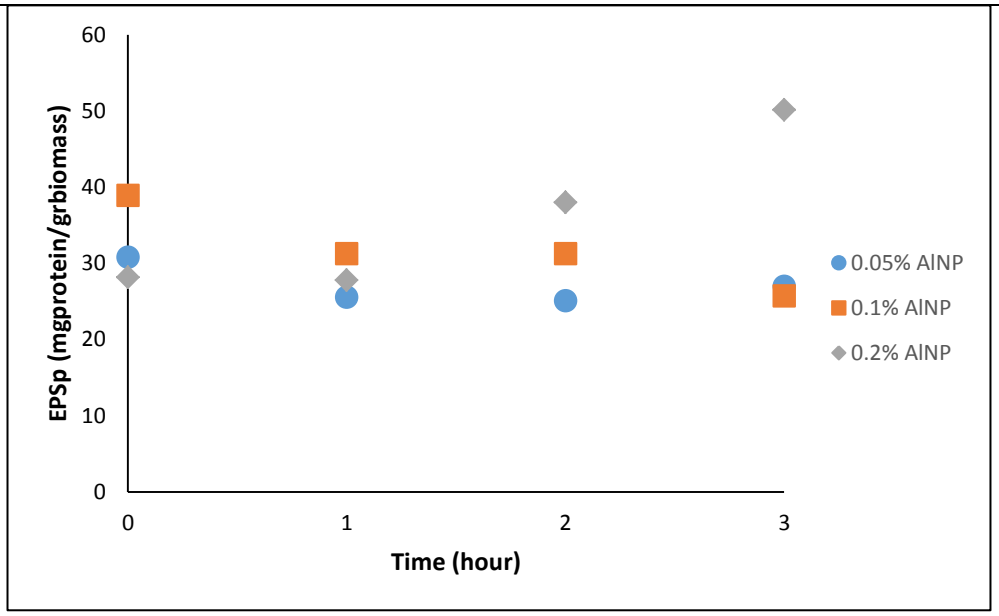
As it can be seen from Figure 4.12(b) that, in the addition of 0.2% AlNPs to activated sludge, EPS_c concentration was increased until 2nd hour, but then decreased back in 3rd hour. For 0.05% AlNPs no significant change and for 0.1% AlNPs a slight decrease were observed in 3 hours period. As a result, it may be said that, the effects of AlNPs on EPSc of the activated sludge is related with nanoparticle ratio.

The overall effects of AlNPs on EPS contents are given in

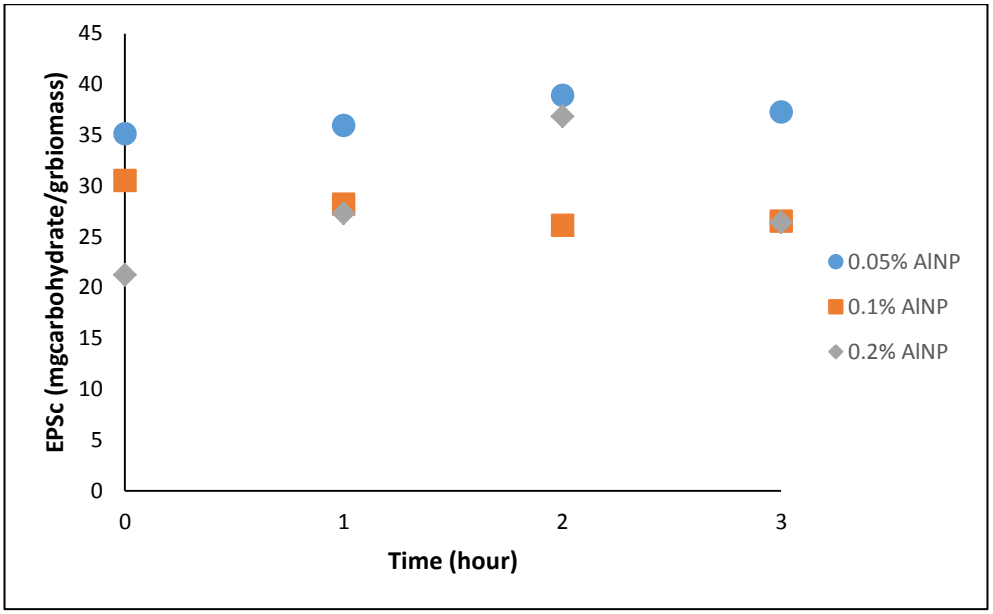
Table 4. **4**. The signs of (+) and (-) shows the increase and decrease, respectively. The highest overall effect for EPS_p was observed as decreasing trend at 0.1% ratio of AlNPs. Furthermore, the highest overall effect for EPS_c was observed as increase trend again at 0.2% ratio of AlNPs.

Table 4. 4 : The values of overall effects of AlNPs on EPS contents.

	Overall effects (%)	
AlNPs ratios (%)	EPS_p	EPS_c
0.05	(-) 14	(+) 6
0.1	(-) 51	(-) 15
0.2	(+) 44	(+) 20



(a)



(b)

Figure 4. 12 : The graphs of EPS analysis at the short-term nanotoxicity test using AlNPs (a) EPS_p (mgprotein/grbiomass) (b) EPS_c (mgcarbohydrate/grbiomass)

4.3 Results of Long-Term Nanotoxicity Tests

Results of long-term nanotoxicity tests are given particularly for AgNP and AlNP as following.

4.3.1 The effect of AgNPs on activated sludge properties

Effects of AgNPs on biological properties of activated sludge were secondly carried out with long-term tests. Optimum conditions according to the dissolution and the short-term experiments were selected for this experiments as pH=7.0, temperature=25.0^{0}C, and three different NPs ratios (0.05-0.1-0.2%, m/m). In the long-term tests, MLSS, viscosity, OD_{sludge}, $OD_{supernatant}$, SMP and EPS analysis were carried out at every day during 10 days experiment.

The graph of MLSS analysis is given in Figure 4. 12. As it can be seen from figure, an increasing trend for MLSS concentration was observed for all AgNP ratios and blank sludge during long-term experiments. As a result it may be said that, AgNPs have no significant effect on MLSS concentration of the activated sludge.

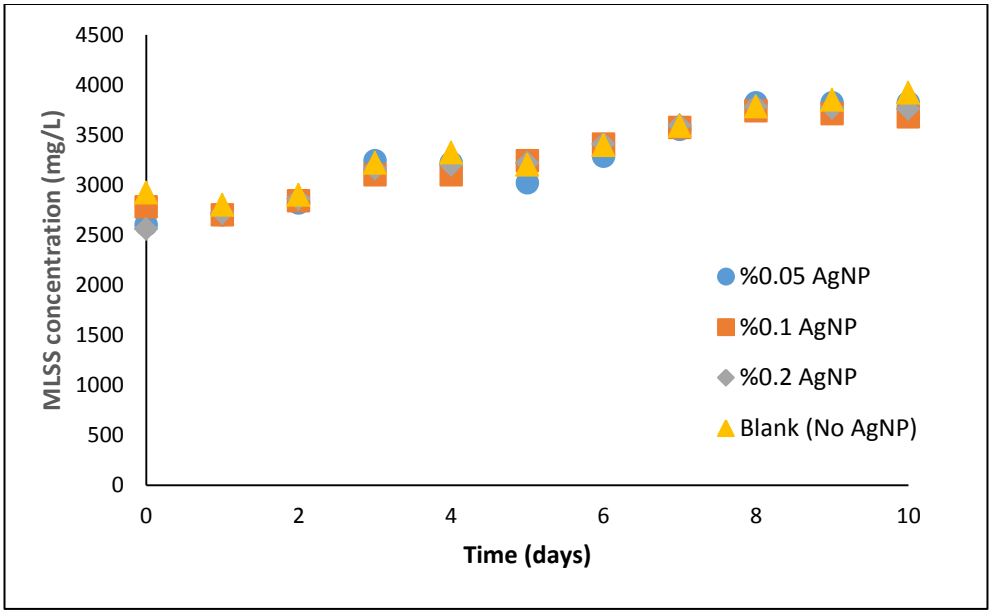


Figure 4. 13 : The MLSS concentration values during AgNPs long-term toxicity experiments.

The graph of viscosity analysis is given in Figure 4. 14. As it can be seen from figure, a similar trend for viscosity was observed at all AgNP ratios and blank sludge during long-term experiments. As a result it may be said that AgNPs did not significantly affect the viscosity values.

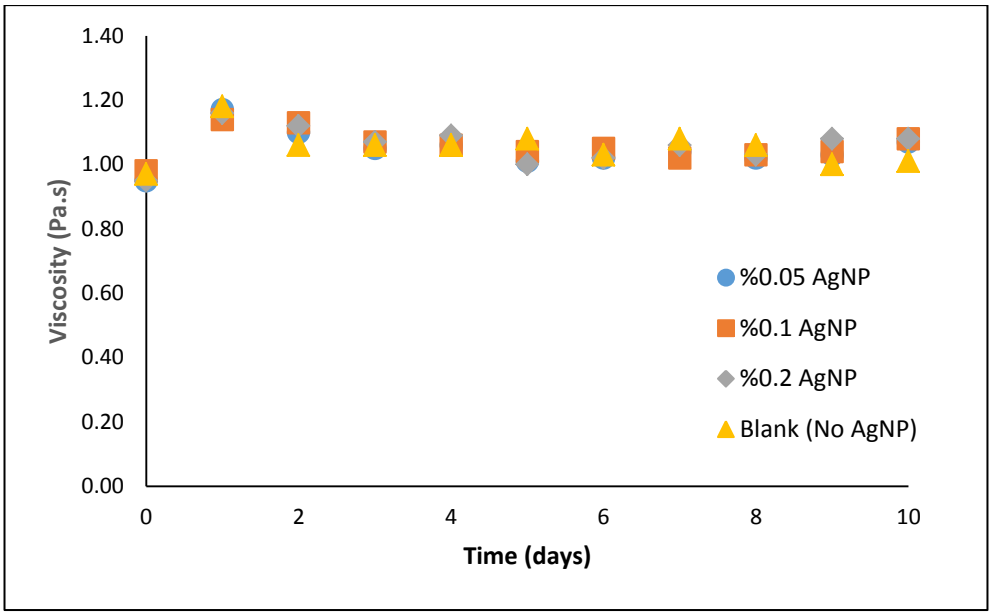


Figure 4. 14 : The viscosity values during AgNPs long-term toxicity experiments.

The graph of OD_{sludge} analysis is given in Figure 4. 15. As it can be seen from figure, a similar trend for OD_{sludge} was observed at all AgNP ratios and blank sludge during long-term experiments. As a result it may be said that AgNPs did not significantly affect the OD_{sludge} values. OD_{sludge} and MLSS concentration values showed similar trends.

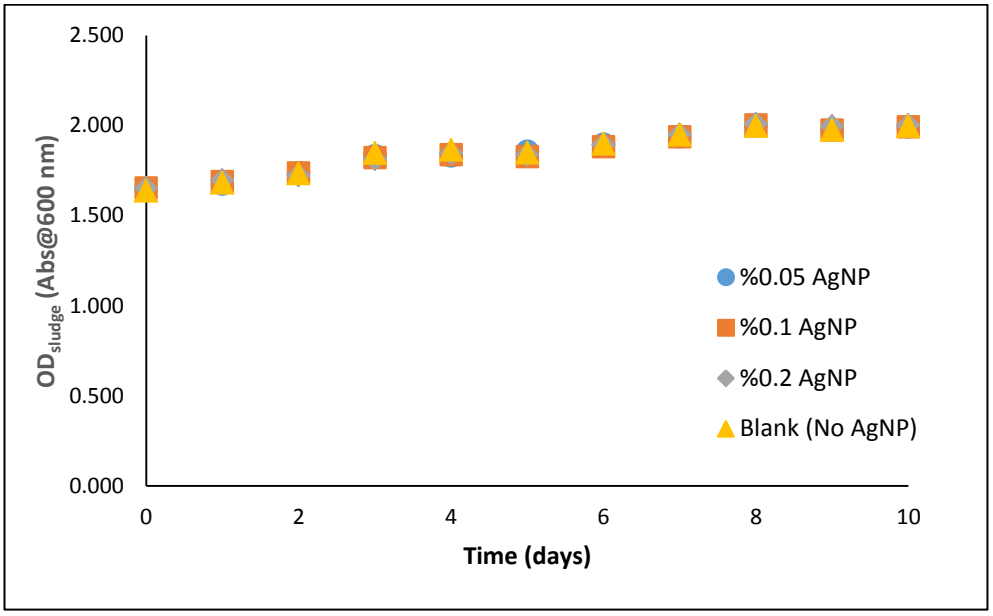
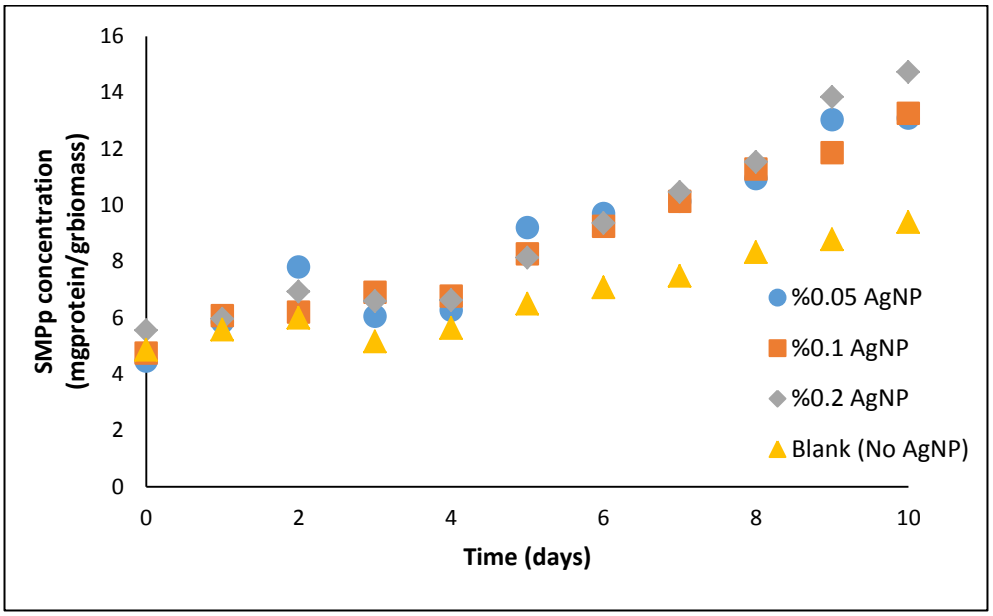


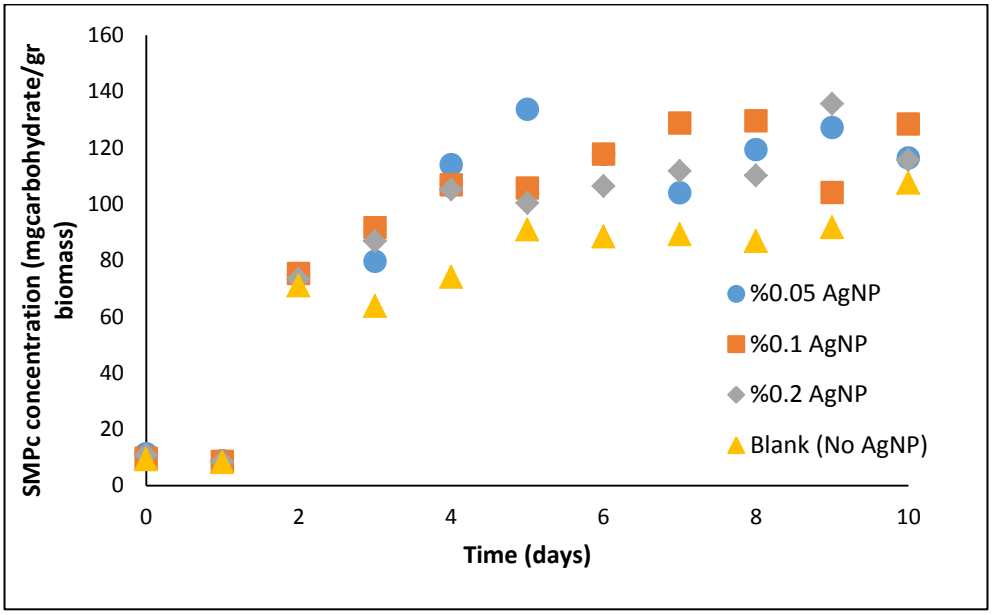
Figure 4. 15 : The OD_{sludge} values during AgNPs long-term toxicity experiments.

The graphs of SMP analysis are given in Figure 4. 16 (a, b). As it can be seen from figures, increasing trends for SMP_p and SMP_c were observed at all AgNP ratios and blank sludge during long-term experiments. However, it can be said that the content

of SMP_p and SMP_c in blank sludge had the lowest values. As a result it may be said that, AgNPs increased the SMP concentrations.



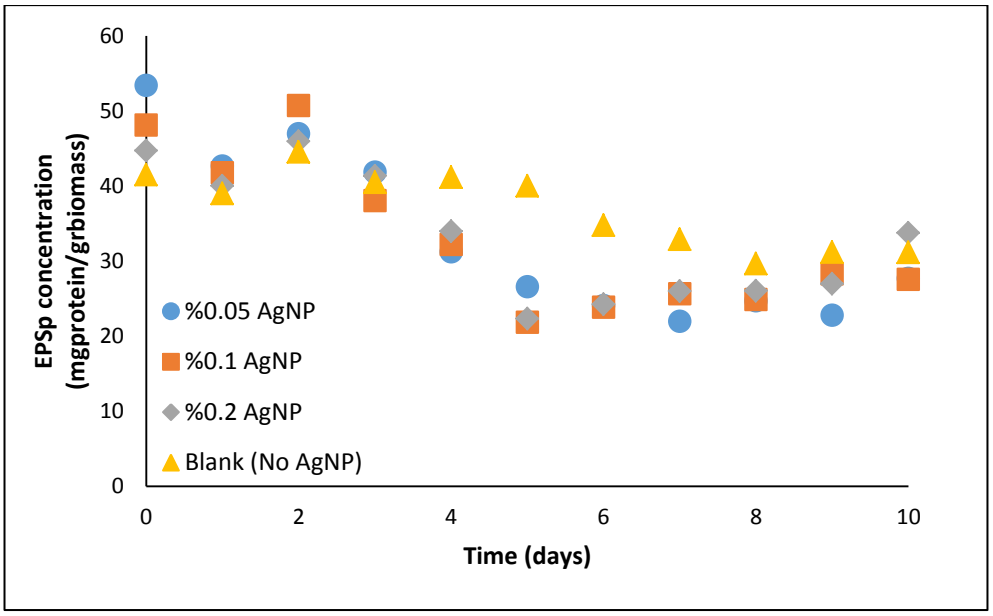
(a)



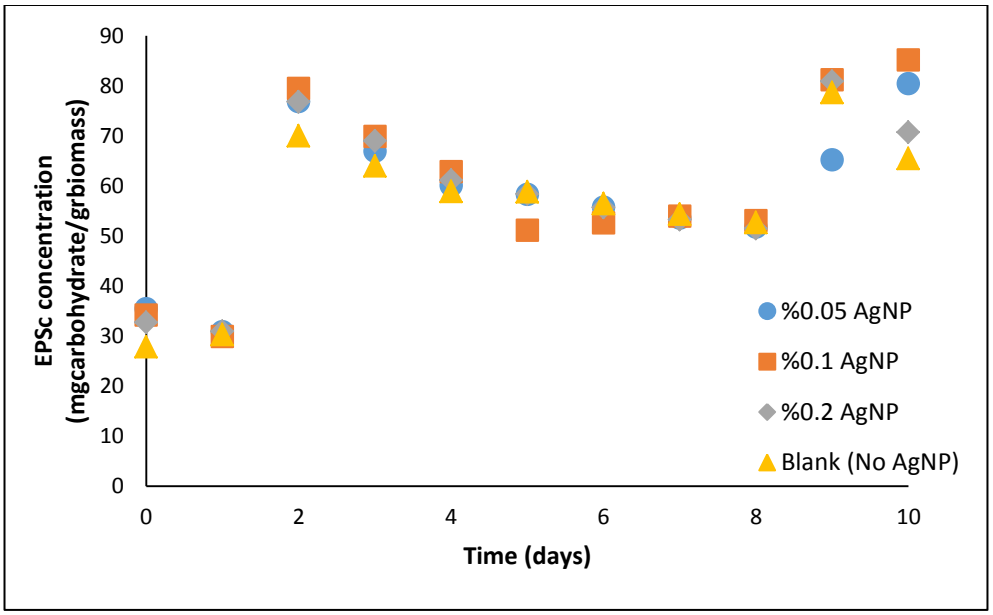
(b)

Figure 4. 16 : The SMP values during AgNPs long-term toxicity experiments (a) SMP_p concentration (mgprotein/grbiomass) (b) SMP_c concentration (mgcarbohydrate/grbiomass)

The graphs of EPS analysis are given in Figure 4. 17 (a, b). As it can be seen from figures, a similar trend for EPS_c was observed at all AgNP ratios and blank sludge during long-term experiments. However, it can be seen that EPS_p was higher in blank sludge than sludge including AgNPs.



(a)



(b)

Figure 4. 17 : The EPS values during long-term toxicity experiments (a) EPS_p concentration (mgprotein/grbiomass) (b) EPS_c concentration (mgcarbohydrate/grbiomass)

The graph of $OD_{supernatant}$ analysis is given in Figure 4. 18. As it can be seen from figures, an increasing trend for $OD_{supernatant}$ was observed at all AgNP ratios and blank sludge during long-term experiments. However, it can be said that $OD_{supernatant}$ in the blank sludge had lower values so this value means that the settling properties of the blank sludge was slightly better than sludge including AgNPs.

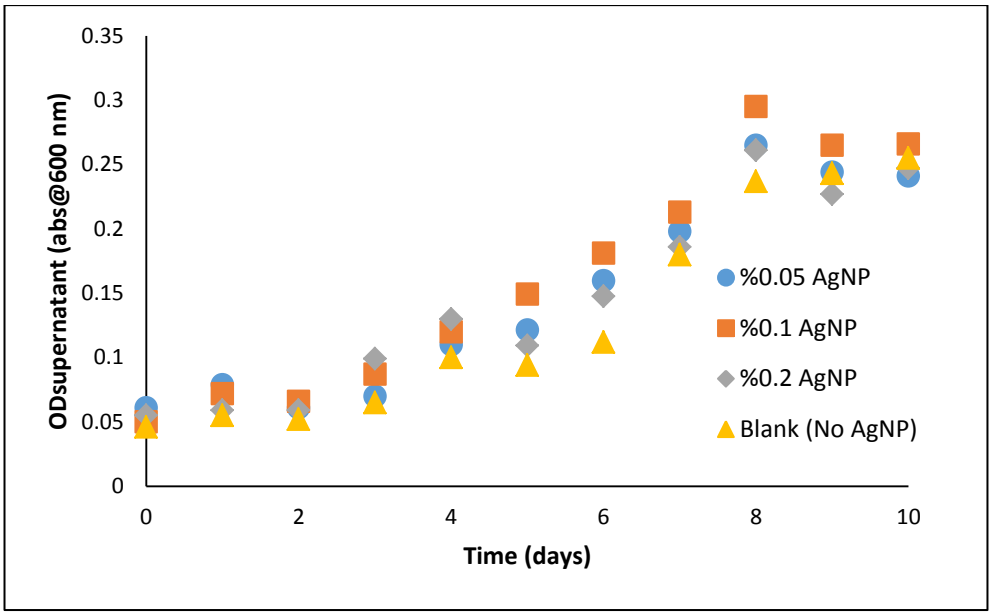


Figure 4. 18 : The $OD_{supernatant}$ values during long-term toxicity experiments.

4.3.2 The effect of AlNPs on activated sludge properties

Effects of AlNPs on biological properties of activated sludge were secondly carried out in long-term tests. Optimum conditions according to the dissolution and the short-term experiments were selected for the experiments as pH=6.3, temperature=25.0^{0}C, and three different NPs ratios (0.05-0.1-0.2%, m/m). In the long-term tests, MLSS, viscosity, OD_{sludge}, $OD_{supernatant}$, SMP and EPS analysis were carried out at every day during 10 days experiment.

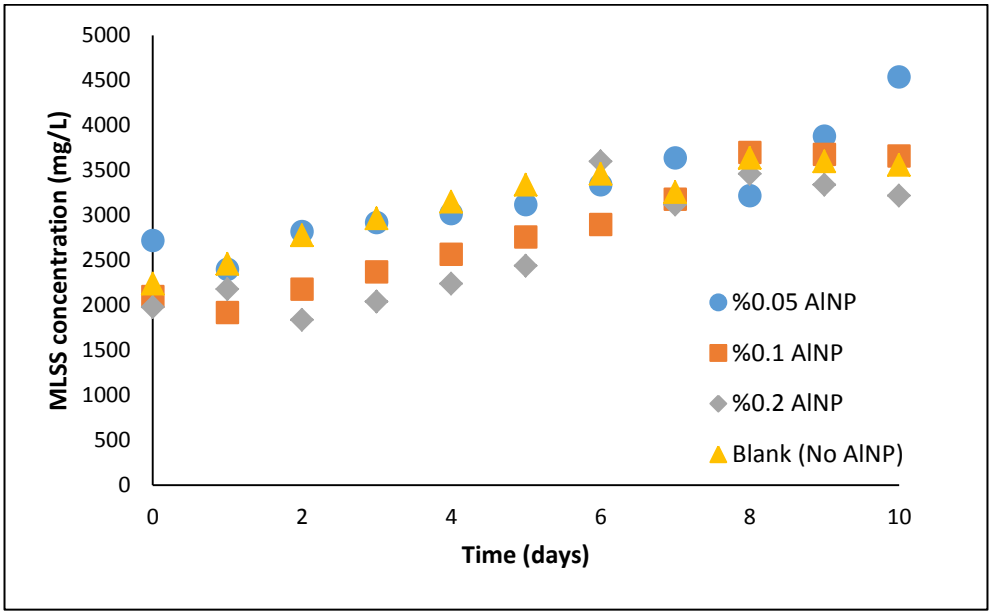


Figure 4. 19 : The MLSS concentration values during AlNPs long-term toxicity experiments.

The graph of MLSS analysis is given in Figure 4. 19. As can be seen from figure, an increasing trend for MLSS concentration was observed at all AlNP ratios and blank sludge during long-term experiments. However, it can be seen that AlNPs decreased the MLSS concentrations and also the bacterial growth until 8th day especially for 0.1 and 0.2% AlNP ratios.

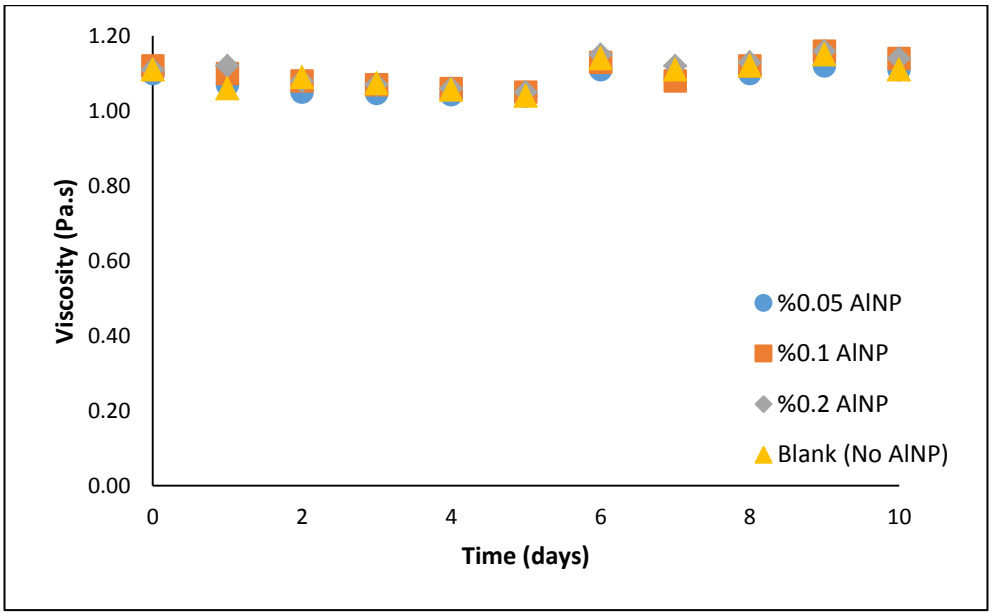


Figure 4. 20 : The viscosity values during AlNPs long-term toxicity experiments.

The graph of viscosity analysis is given in Figure 4. 20. As it can be seen from figure, a similar trend for viscosity was observed at all AlNP ratios and blank sludge during long-term experiments. It can be said that AlNPs did not significantly affect the viscosity values of the sludge.

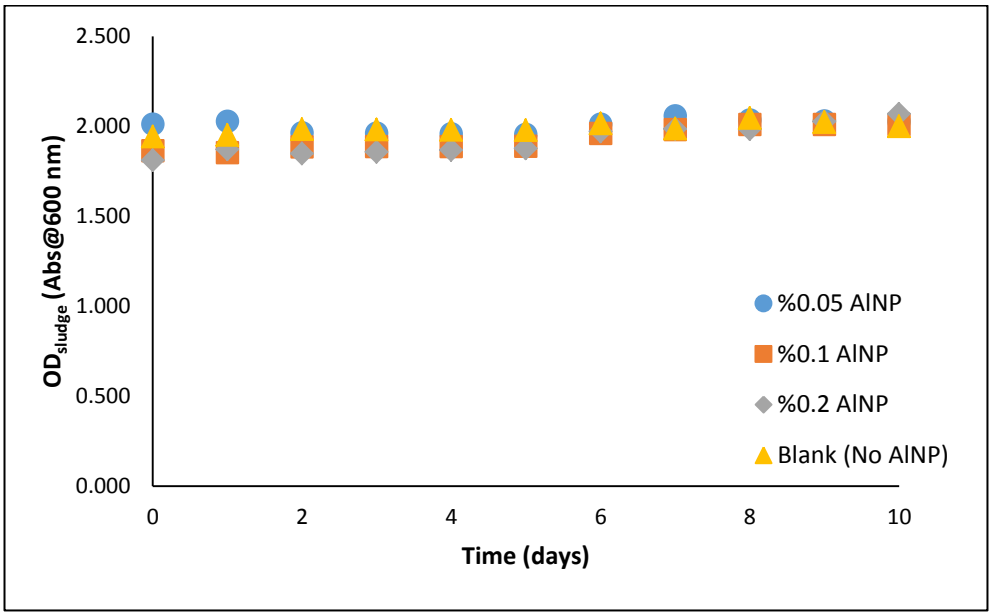
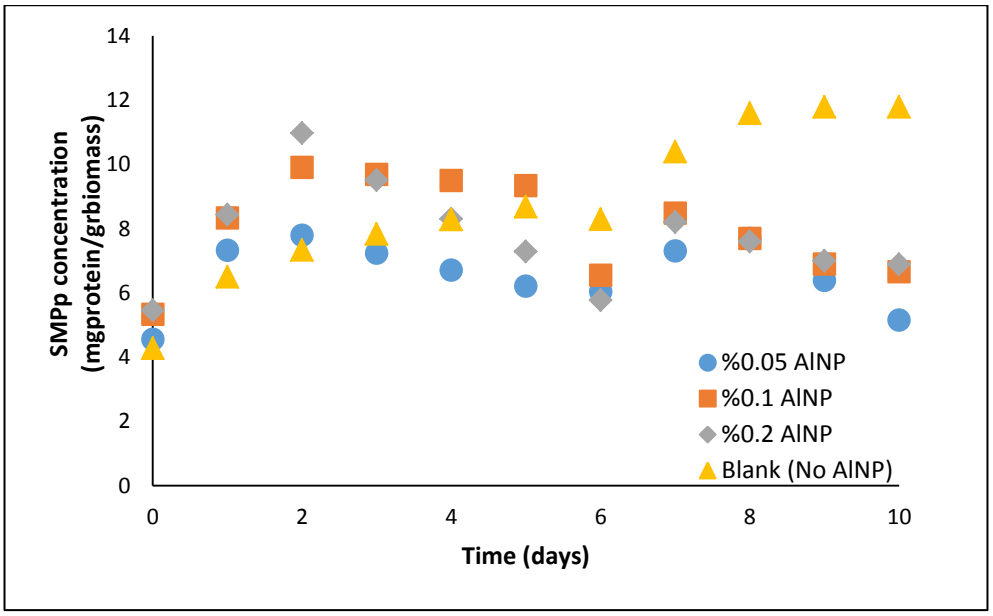
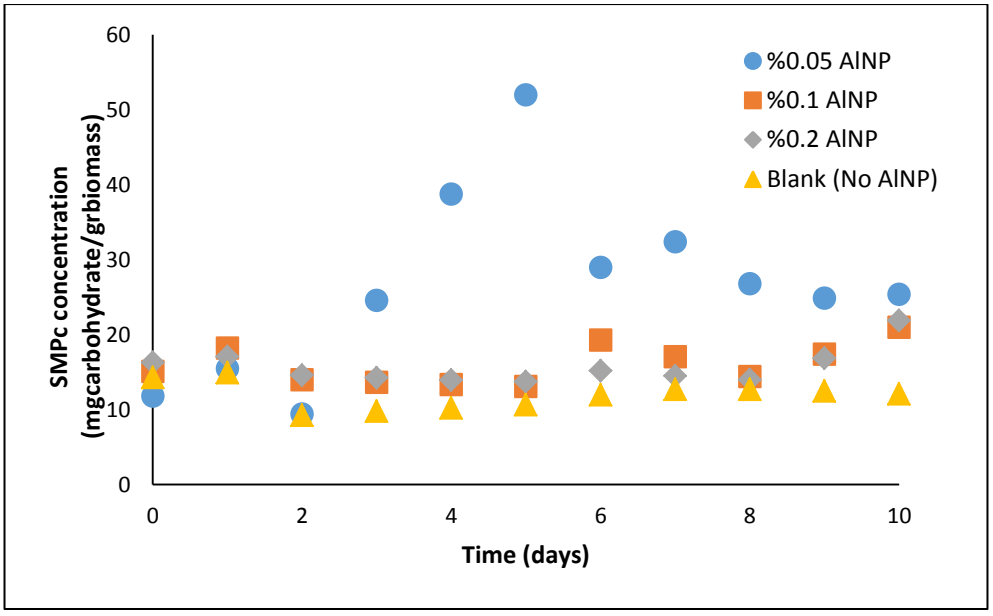


Figure 4. 21 : The OD$_{sludge}$ values during AlNPs long-term toxicity experiments.

The graph of OD_{sludge} analysis is given in Figure 4. 21. As it can be seen from the figure, a similar trend for OD_{sludge} was observed at all AlNP ratios and blank sludge during long-term experiments. It can be said that AlNPs did not significantly affect the OD_{sludge} values but also OD_{sludge} and MLSS concentration values showed similar trend.



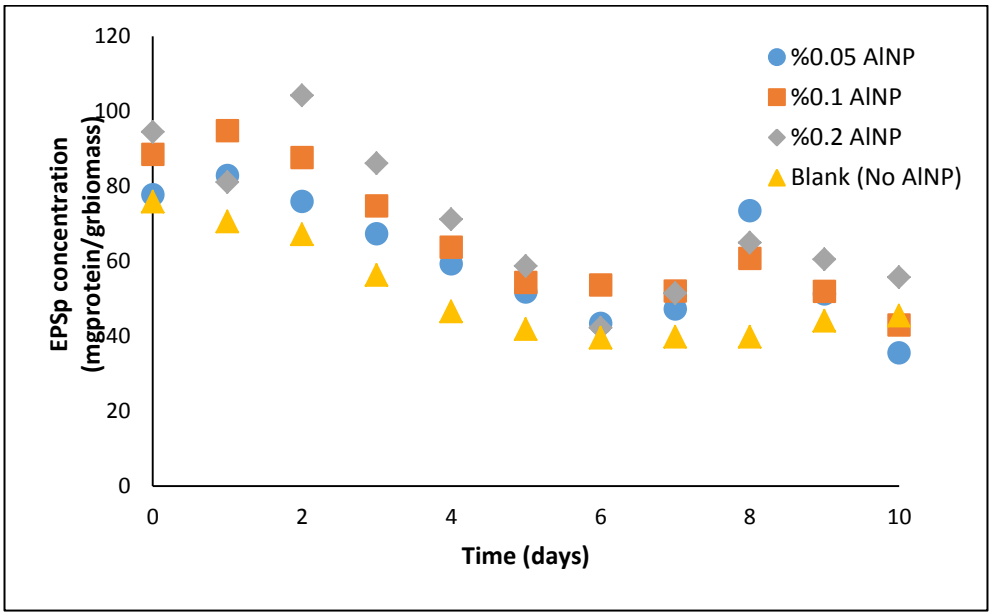
(a)



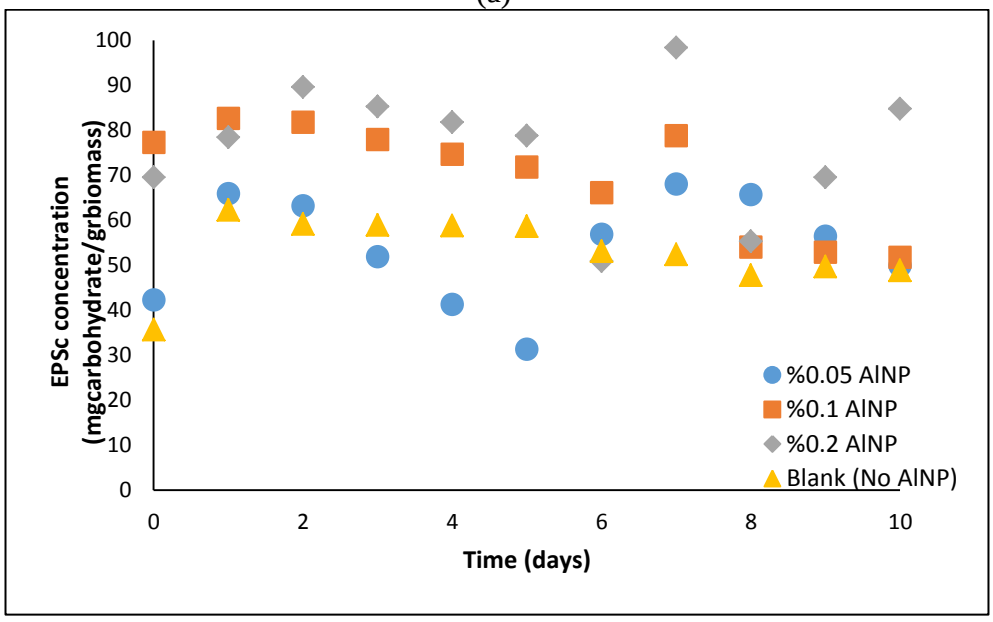
(b)

Figure 4. 22 : The SMP values during AlNPs long-term toxicity experiments SMPp concentration (mgprotein/grbiomass) (b) SMPc concentration (mgcarbohydrate/grbiomass)

As it can be seen from figures, an increasing trend for SMP_p were observed at all AlNP ratios and blank sludge until 2nd day. After that, the SMP_p concentrations had reached to constant values on the 7th day, a different trend was observed at blank sludge and sludge with AlNPs. SMP_p concentrations of the blank sludge increased while it decreased at other sludges with AlNP. From Figure 4.22 (b), SMP_c concentration at sludge including 0.05% AlNP reached to the highest values while the blank sludge had the lowest SMP_c concentrations.



(a)



(b)

Figure 4. 23 : The EPS values during AlNPs long-term toxicity experiments (a) EPSp concentration (mgprotein/grbiomass) (b) EPSc concentration (mgcarbohydrate/grbiomass)

The graph of EPS analysis is given in Figure 4. 23. As it can be seen from figures, similar trends for EPS_p and EPS_c were observed at all AlNP ratios and blank sludge during long-term experiments. However, it can be seen that EPS concentrations were higher in the sludge including AlNPs than the blank sludge. Thus, it can be concluded that AlNP increases both EPS_p and EPS_c concentrations.

As it can be seen from figure, an increasing trend for $OD_{supernatant}$ was observed for all AlNP ratios and the blank sludge during long-term experiments. However, it can be said that 0.1% AlNPs had higher $OD_{supernatant}$ values than others.

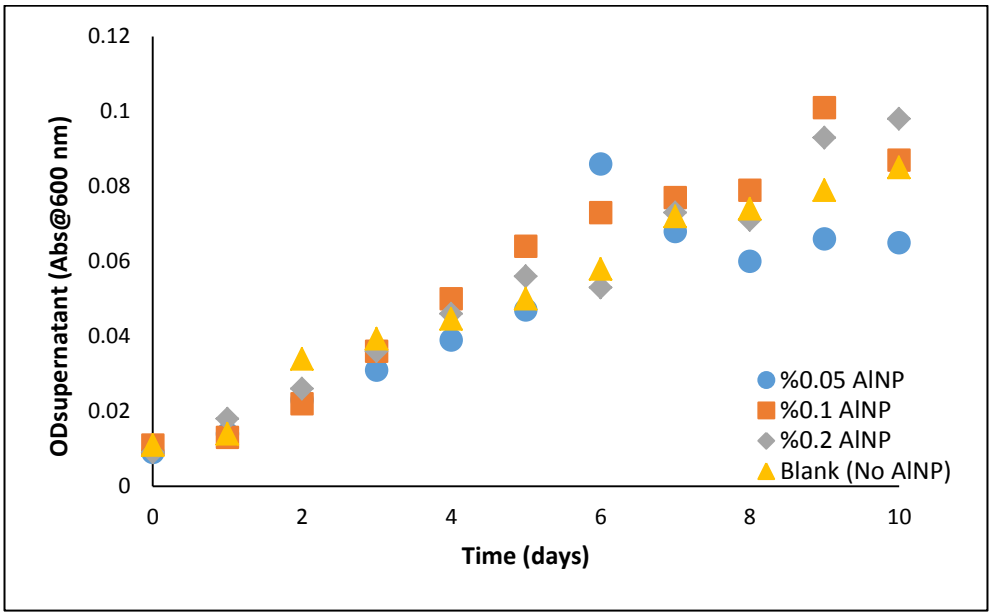


Figure 4. 24 : The ODsupernatant values during AlNPs long-term toxicity experiments.

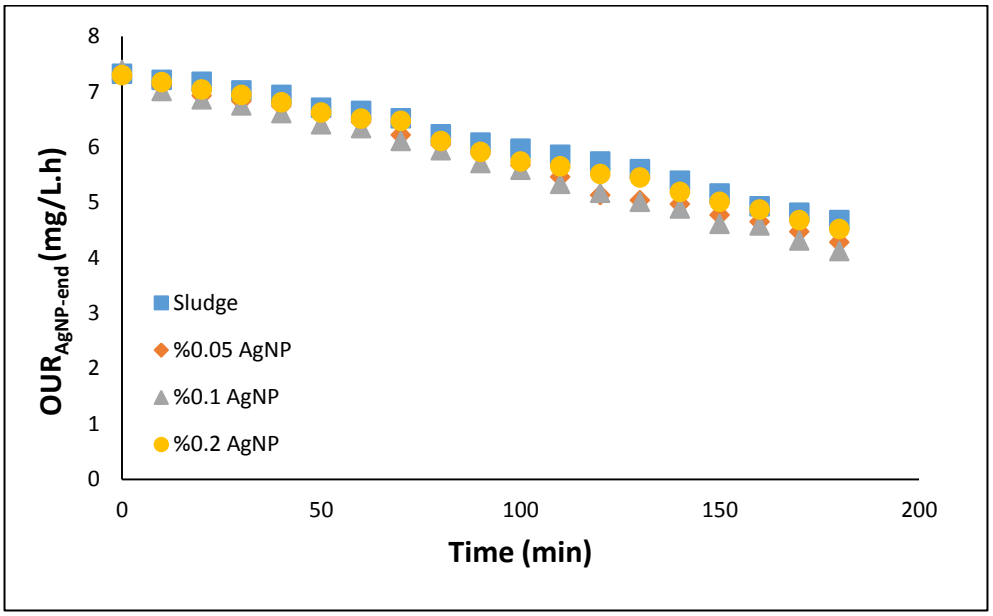
4.4 Results of Respirometric Nanotoxicity Tests

Results of respirometric nanotoxicity tests are particularly given for AgNP and AlNP as following.

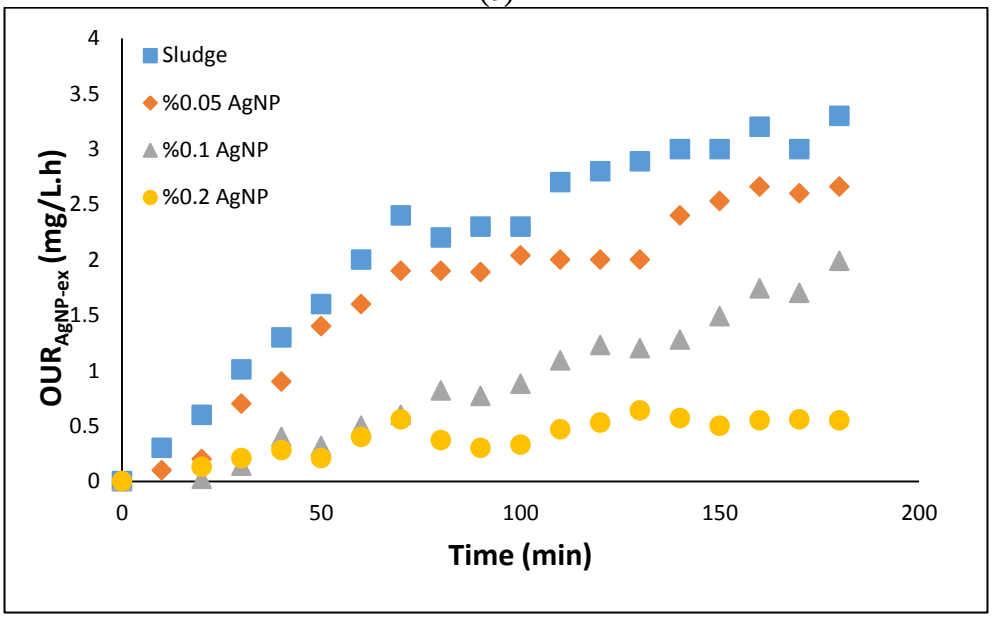
4.4.1 The effect of AgNPs on respirometric experiments

Effects of AgNPs on respirometric activity of activated sludge were carried out with respirometric tests. Therefore, the inhibition rates were also calculated from this experiment. The optimum conditions according to pre-experiments were selected for the experiments as pH=7.0, temperature=25.0^{0}C and three different NPs ratios (0.05-0.1-0.2%, m/m). In the experiments, dissolved oxygen (DO) analysis were carried

out at every 10-min during 3-h experiment. MLSS concentration of activated sludge samples were averagely found as 2857±86 mg/L.



(a)



(b)

Figure 4. 25 : The respirometric parameters of AgNP on respirometric experiments (a) $OUR_{AgNP\text{-}end}$ (b) $OUR_{AgNP\text{-}ex}$

Graphs of the oxygen uptake rate for endogenous phase ($OUR_{AgNP\text{-}end}$) and for exogeneous (substrate utilaization) phase ($OUR_{AgNP\text{-}ex}$) are given in Figure 4. 25(a, b). All $OUR_{AgNP\text{-}end}$ values decreased linearly. 0.1% AgNP had the lowest values. From the figure it can be said that, the addition of AgNPs decreased the endogeneous respiration ratio in the activated sludge. In Figure 4.25(b), the $OUR_{AgNP\text{-}ex}$ graph can be seen both for blank and sludge having AgNPs. The blank sludge had the highest

OUR_{ex} values while the sludge having AgNPs had lower values. Especially, the sludge having 0.2%AgNP had the lowest $OUR_{AgNP\text{-}ex}$ values. These results shows that, the AgNPs affect the oxygen uptake mechanism of microorganisms in activated sludge.

Figure 4. 26 showed the inhibition ratios of AgNPs depending on its amounts. The inhibition ratios were found as 14.4, 34.8 and 81.9% for 0.05% AgNP, 0.1% AgNP and 0.2% AgNP, respectively. As the AgNPs ratios are increased, the inhibition rate increases. This means that AgNPs inhibites the microorganisms via oxygen uptake mechanism.

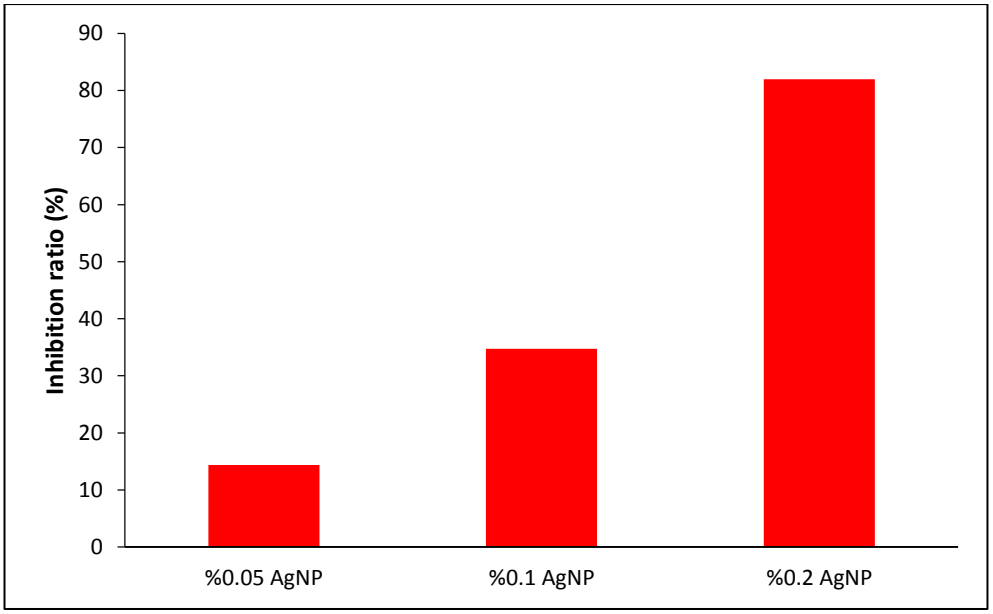


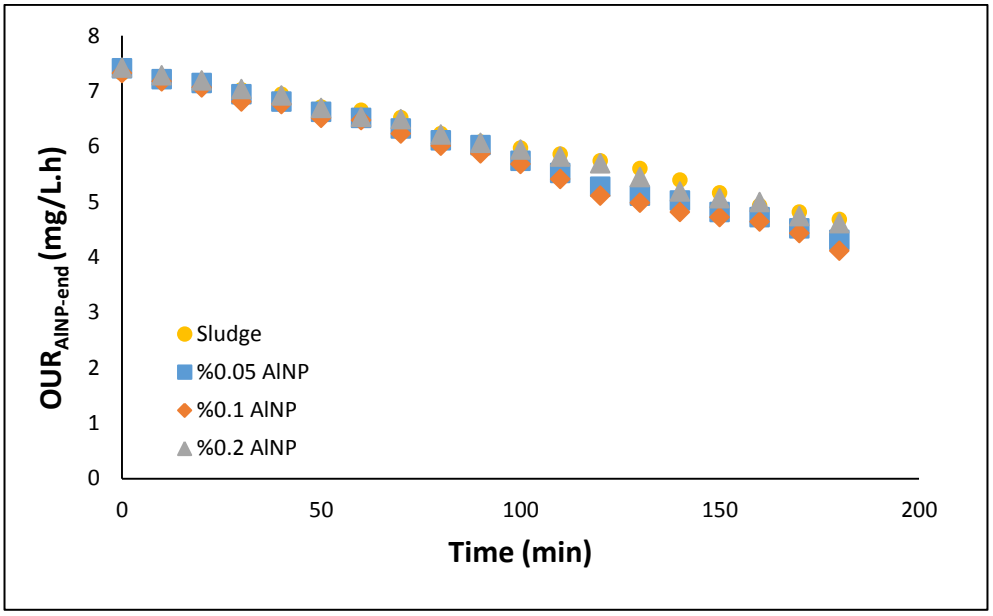
Figure 4. 26 : The inhibition ratio of AgNPs calculated from OUR measurements.

From the respirometric analysis of AgNPs, it can be concluded that AgNPs dramatically affects the oxygen utilization mechanism of microorganisms in activated sludge.

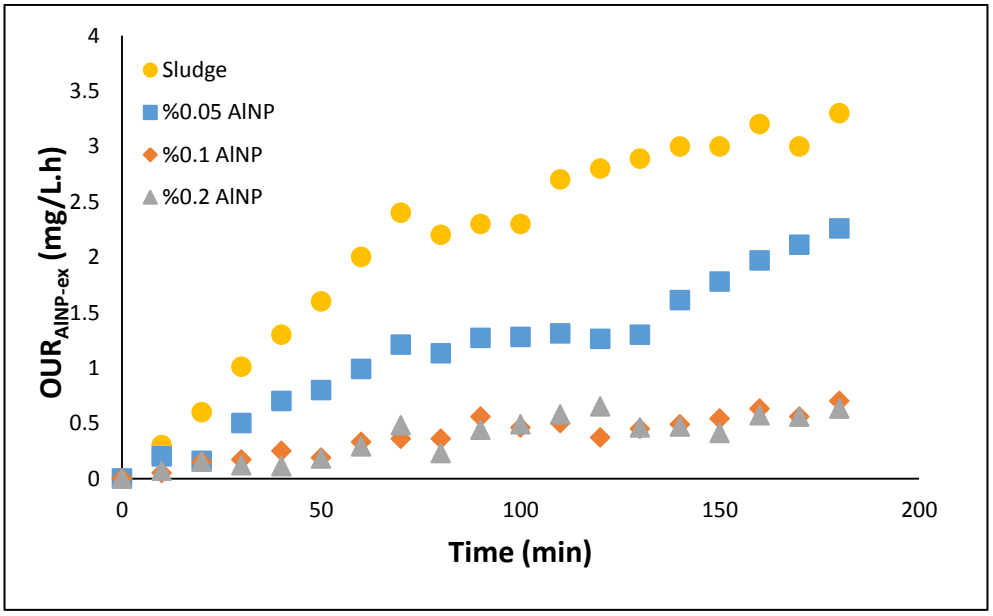
4.4.2 The effect of AlNPs on respirometric experiments

Effects of AlNPs on respirometric activity of activated sludge were carried out with respirometric tests. Therefore, the inhibition rates were also calculated from this experiment. The optimum conditions according to pre-experiments were selected for the experiments as pH=6.3, temperature=25.0^{0}C and three different NPs ratios (0.05-0.1-0.2%, m/m). In the experiments, dissolved oxygen (DO) analysis were carried out at every 10-min during 3 h experiment. MLSS concentration of activated sludge samples were averagely found as 2613±23 mg/L. Graphs of the oxygen uptake rate at endogenous phase ($OUR_{AlNP\text{-}end}$) and at exogeneous (substrate utilization) phase

($OUR_{AlNP\text{-}ex}$) are given in Figure 4. 27(a, b). All $OUR_{AlNP\text{-}end}$ values decreased linearly but 0.1% AlNP had the lowest values (similar to AgNPs). From the figure, it can be said that the addition of AlNPs slightly decreased the endogeneous respiration ratio in sludge. In Figure 4.27(b), the $OUR_{AlNP\text{-}ex}$ graphs can be seen for both blank and having AlNPs sludge. The blank sludge had the highest OUR_{ex} values while the sludge having AlNPs had lower values. Especially the sludge having 0.2% AlNP and 0.1% AlNP had the lowest $OUR_{AlNP\text{-}ex}$ values. These results showes that, AlNPs affect the oxygen uptake mechanism of microorganisms in activated sludge.



(a)



(b)

Figure 4. 27 : The respirometric parameters of AlNP on respirometric experiments (a) $OUR_{AlNP\text{-}end}$ (b) $OUR_{AlNP\text{-}ex}$

Figure 4. 28 shows the inhibition ratios of AlNPs depending on its amounts. The inhibition ratios were found as 34.8, 80.8 and 80.8% for 0.05%AlNP, 0.1%AlNP and 0.2%AlNP, respectively. As AlNP ratios are increased, the inhibition rate increases. This means that AlNPs inhibites the microorganisms via oxygen uptake mechanism.

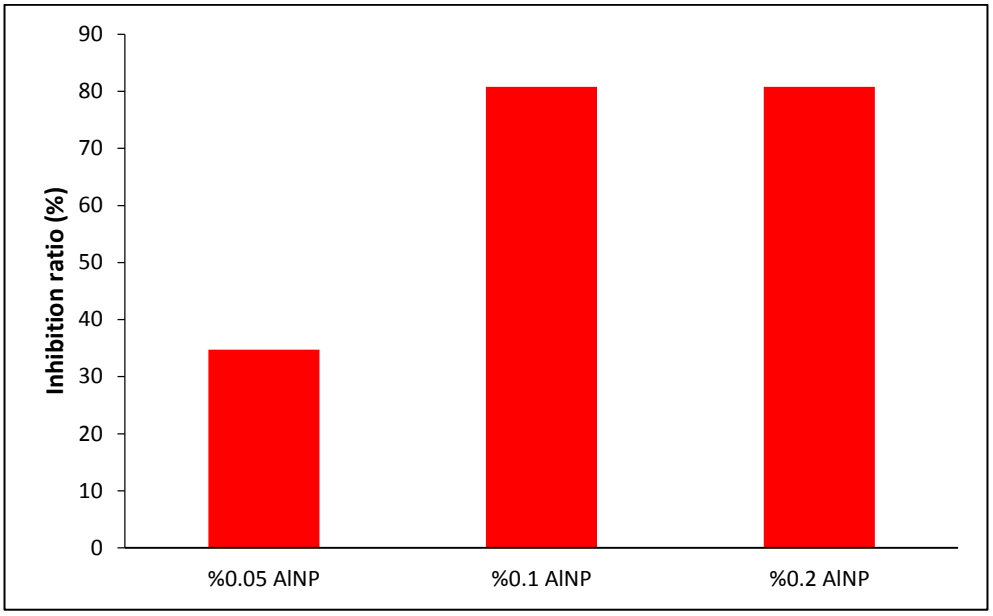


Figure 4. 28 : The inhibition ratio of AlNPs calculated from OUR measurements.

5. CONCLUSIONS

The aim of this work was to determine the nanotoxicological effects of AgNP and AlNP on microbial community structure in activated sludge. The contributions for determination of toxicological effects were examined under three main title as dissolution experiments, short term shock load experiments and long term experiments.

In the first stage of experiments, dissolution of the maximum dissolution was observed at 0.1% AgNP ratio and so the optimum AgNP ratio was selected as 0.1%. in distlled water were determined for pH (3 - 5 - 7), temperature (25^oC – 35^oC – 45^oC), stirring rate (200 rpm – 400 rpm – 600 rpm – 800 rpm), nanoparticle concentrations (0.1 - 0.2 - 0.6 - 1%). Because of proportional dissolution rates both for AgNP and AlNP, results were annotated collectively.

In the pH experiments, three different pH values (3.0, 5.0 and 7.0) were tested while the temperature (at 25^0C), stirring rate (at 200 rpm) and NP ratio (at 0.4%, m/m) were constant. The maximum dissolution values were observed at pH 7.0 for AgNP and pH 3.0 for AlNP. According to those analysis, the optimum pH was determined as pH 7.0 which has no damaging effect for activated sludge bacterial cells.

In the temperature experiments, three different temperature values (25.0, 35.0 and 45.0^0C) were tested while the pH (at 7.0), stirring rate (at 200 rpm) and NP ratio (at 0.4%, m/m) were constant. The dissolution was decreased with increasing temperature and is maximum at room temperature for AgNP, but not affected remarkably for AlNP. As a result, optimum temperature was determined as 25^0C for both NP types.

In the striring rate experiments, four different stirring rate values (200, 400, 600 and 800 rpm) were tested while the pH (at 7.0), temperature (at 25^0C) and NP ratio (at 0.4%, m/m) were constant. The dissolution ratio was increased with increasing stirring rate from 200 rpm to 400 rpm but then it decreased with increasing stirring rate for AgNP and decreased with increasing stirring rate from 200 rpm to 400 rpm

and no effect was observed for stirring rates higher than 400 rpm for AlNP. As a result, the optimum stirring rate was determined as 400 rpm for AgNP and 200 rpm for AlNP.

In the NPs ratio experiments, four different NPs ratio values (0.1, 0.2, 0.6 and 1.0%) were tested while the pH (at 7.0), temperature (at 25^0C) and stirring rate (at 400 rpm) were constant. The dissolutions were dramatically decreased with increasing NP ratio for both AgNPs and AlNPs. It can be concluded that, the increasing concentration of NPs ratio may cause agglemoration of nanoparticles in solution. So, the dissolution efficiency was declined. Maximum dissolutions were observed at 0.1% AgNP ratio. As a result, the optimum NP ratio was determined as 0.1%. By reason of low dissolution ratios for high concentrations, 0.05% NP concentration is added for following experiments.

In the second stage of experiments, effects of AgNPs and AlNPs on biological properties of activated sludge were carried out with shock load analysis. In the short-term tests, SMP and EPS analysis were carried out at every hour during 3 h experiment.

For AgNP;

MLSS concentration of activated sludge samples were averagely measured 2267±76 mg/L.

The effects of AgNPs on SMP_p of the activated sludge is related with nanoparticle ratio. The addition of 0.05% AgNP to the activated sludge, has no significant affect for SMP_p. However, when 0.1% AgNP was added, an increasing trend was observed during 3-h experiment. When 0.2% AgNP was added to activated sludge, SMP_p maked a peak in the 2^{nd} hour, then decreased back to its initial values.

The SMP_c of the activated sludge is not affected due to AgNPs ratio at the end of 3-h period. The addition of 0.05, 0.1 and 0.2% AgNPs to activated the sludge, has no affect on SMP_c. For 0.1% AgNPs, a slight increase was observed at 1^{st} and 3^{rd} hours, but in the 2^{nd} hour it turned to its normal values.

The EPSp of the activated sludge is not affected with AgNPs ratio. The addition of 0.05% AgNP to the activated sludge had a significant affect for EPS_p, but when 0.1 and 0.2% AgNP were added, a similar trend was observed during 3-h experiment. The concentration of EPSp was the highest at 0.05% AgNP.

The EPSc of the activated sludge is increased due to increasing AgNPs ratio.

For AlNP;

MLSS concentration of activated sludge samples were averagely measured 2847±70 mg/L.

The SMPp of the activated sludge is not affected due to AlNPs ratio. Increasing trends were observed for all of the AlNP ratios in the activated sludge in the first hour but then SMP_p concentrations were decreased.

The SMPc of the activated sludge is not affected due to AlNPs ratio. The addition of 0.05, 0.1 and 0.2% AlNP to activated sludge, has no significant affect on SMP_c.

The effects of AlNPs on EPSp of the activated sludge is related with nanoparticle ratio. In the addition of 0.2% AlNPs to the activated sludge, an increasing effect for EPS_p was observed but when 0.1 and 0.05% AlNPs were added, decreasing trends were observed during 3-h experiment.

The effects of AlNPs on EPSc of the activated sludge is related with nanoparticle ratio. In the addition of 0.2% AlNPs to activated sludge, EPS_c concentration was increased until 2^{nd} hour, but then decreased back in 3^{rd} hour. For 0.05% AlNPs no significant change and for 0.1% AlNPs a slight decrease were observed in 3 hours period.

In the third stage of experiments, effects of NPs on biological properties of activated sludge were secondly carried out with long-term tests. The optimum conditions were selected same as short-term experiments.

For AgNP;

AgNPs have no significant affect on MLSS concentration of the activated sludge.

AgNPs did not significantly affect the viscosity values.

AgNPs did not significantly affect the OD_{sludge} values but also OD_{sludge} and MLSS concentration values showed similar trend.

AgNPs did not significantly affect the OD_{sludge} values but also OD_{sludge} and MLSS concentration values showed similar trend.

AgNPs increased the SMP_p and SMP_c concentrations.

EPS_p was higher in blank sludge than sludge including AgNPs. However, similar trend for EPS_c were observed at all AgNP ratios and blank sludge during long-term experiments.

$OD_{supernatant}$ in blank sludge had lower values so this value meant that the settling properties of blank sludge was slightly better than sludge including AgNPs.

For AlNP;

AlNPs decreased the MLSS concentration and so the bacterial growth especially at 0.1 and 0.2% AlNP ratios.

AlNPs did not significantly affect the viscosity values.

AlNPs did not significantly affect the OD_{sludge} values but also OD_{sludge} and MLSS concentration values showed similar trends.

An increasing trend for SMP_p was observed for all AlNP ratios and blank sludge until 2nd day. After that, the SMP_p concentrations reached to constant values. After the 7th day, a different trend was observed at blank sludge and sludge with AlNP. SMP_p concentration of the blank sludge increased while it decreased at the sludge with AlNP. SMP_c concentration at sludge including 0.05% AlNP reached to the highest values while the blank sludge had lowest SMP_c concentrations.

Similar trends for EPS_p and EPS_c were observed at all AlNP ratios and blank sludge during long-term experiments. However, EPS concentrations were higher in the sludge including AlNPs than blank sludge. Thus, it can be concluded that AlNP increased both EPS_p and EPS_c concentrations.

An increasing trend for $OD_{supernatant}$ was observed at all AlNP ratios and blank sludge during long-term experiments. However, it can be said that 0.1% AlNPs had higher $OD_{supernatant}$ values than other sludges.

Effects of NPs on respirometric activity of activated sludge were carried out with respirometric tests and the inhibition rates were also calculated.

For AgNP;

The MLSS concentration of activated sludge samples were averagely found as 2857±86 mg/L

AgNPs decreased the endogeneous respiration ratio in sludge. The results show that the AgNPs affect the oxygen uptake mechanism of microorganisms in activated sludge. The increasing of AgNP ratios increased the inhibition rate. This means that the AgNPs inhibite the microorganisms via oxygen uptake mechanism.

For AlNP;

The MLSS concentration of activated sludge samples were averagely found as 2613±23 mg/L.

AlNPs slightly decreased the endogeneous respiration ratio in sludge. The results shows that the AlNPs affect the oxygen uptake mechanism of microorganisms in activated sludge. The increasing of AlNP ratios increased the inhibition rate. This means that the AlNPs inhibite the microorganisms via oxygen uptake mechanism.

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