ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

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M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

İKİ FARKLI SAF KÜLTÜR VE AKTİF ÇAMUR ÜZERİNDE TRIKLOSAN TOKSİSİTESİNİN BELİRLENMESİ

YÜKSEK LİSANS TEZİ

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OCAK 2013

Eren GÖRENOĞLU, a M.Sc. student of ITU Graduate School of Science Engineering and Technology 501091818 successfully defended the thesis entitled **"DETERMINATION OF TRICLOSAN TOXICITY ON TWO PURE CULTURES AND ACTIVATED SLUDGE"**, which he/she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : **17 December 2012**
Date of Defense : **24 January 2013**

FOREWORD

I would like to thank to my thesis supervisor Assoc. Prof. Dr. Elif PEHLİVANOĞLU MANTAŞ for her guidance, valuable advices, patience and encouragement for every stage of the study. I am very honored for taking place in her harmonious project group and for learning scientific decisiveness from her that is going to be a guide throughout my life.

I would also kindly thank to Prof. Dr. Emine UBAY ÇOKGÖR for her contribution to conducting experimental studies and interpretation of the results.

Special thanks to Res. Assis. Dr. Egemen AYDIN for his guidance for proceeding experiments and sharing his experiences throughout the study.

I would also like to thank Res. Assis. Emel TOPUZ for sharing her laboratory knowledge and supporting the experiments virtually.

I want to thank to my homemate Res. Assis. Bora AKAYDIN for being patient, helpful and tolerant.

Finally I want to thank to my parents for their love and understanding.

This study was supported by ITU BAP No: 34164

January 2013 Eren GÖRENOĞLU

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ABBREVIATIONS

2,8 DCDD	: 2,8-dichlorodibenzo-p-dioxin
BOD	: Biological oxygen demand
CAS	: Chemical Abstracts Service
DOM	: Dissolved organic matters
ENR	: Enoyl-acyl reductase
EPA	: Environmental Protection Agency
EU	: European Union
HS	: Humic substance
MIC	: Maximum inhibitory concentration
MLSS	: Mixed liqueur suspended solid
MRSA	: Methicillin resistant Staphylococcus aureus
MW	: Molecular weight
NAD	: Nicotinamide adenine dinucleotide
NOEC	: No observed effect concentration
OECD	: Organisation for Economic Cooperation and Development
OUR	: Oxygen utilization rate
PCPP	: Personal care products and preservatives
RLU	: Relative Lightning Unit
SOUR	: Specific oxygen utilization rate
SVI	: Sludge volume index
TCS	: Triclosan
TOC	: Total organic carbon
USFDA	: United States Food and Drug Agency
WWTP	: Wastewater treatment plant

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DETERMINATION OF TRICLOSAN TOXICITY ON TWO PURE CULTURES AND ACTIVATED SLUDGE

SUMMARY

Triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol: TCS] is a halogenated phenol and a broad spectrum antimicrobial agent that is used in personal care products and pharmaceuticals (PCPP) such as soaps, toothpaste, mouthwash, deodorant, and cosmetics as well as in textiles nearly for 40 years. Concentrations of TCS in PCPPs are typically 0.1-0.3% and it is a high production volume chemical according to OECD.

After human consumption, TCS reaches wastewater treatment plants where it is subjected to biological treatment and the untreated portion of TCS will be discharged to receiving waters. TCS concentration is reported to range mostly between 0.5-30 μg/L in wastewater. Nevertheless, there are studies which measured concentrations up to 0.6 mg/L. As an antibacterial agent, the main effect of TCS is its acute toxicity on organisms.

Since most of the TCS will be subjected to biological treatment, the first experiment to determine its toxicity was conducted on activated sludge biomass by using oxygen uptake rate. The studies indicated that TCS shows non-competitive inhibition effect to the heterotrophic biomass at concentrations around 6.0 mg/L by inhibiting lipid synthesis and almost no effect was observed at concentration of 0.8 mg/L. One marine bacteria, marine algae and freshwater algae was selected to further study the effects of TCS in receiving waters. The bacterial toxicity tests were conducted on the bioluminescence marine bacteria *Vibrio fisheri*. *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum* were selected as freshwater and seawater algae respectively as the unicellular algal species to conduct growth inhibition test (EN ISO 8692:2004). The EC_{50} concentration for 15- min bacterial luminescence bioassays was determined as 0.67 mg/L whereas the EC_{50} value for algal growth inhibition was 7.74 μg/L. Moreover, the effect of direct and indirect photolysis on the toxicity of TCS was studied in the absence and presence of humic substances, respectively. The EC_{50} value for *V. fischeri* after photodegradation was 1.29 mg/L which was confirmed with LC-MS/ MS measurements where 40% decrease in the signal intensity of TCS was observed. After indirect photolysis (presence of humic substance) the EC_{50} value was increased to 1.37 mg/L. EC_{50} value for *P. subcapitata* after direct photolysis of TCS was found as 8.40 μg/L. Moreover our study was not detected 2,4 DCP by LC-MS/MS measurements as proposed photoproduct of TCS.

These results indicate that the concentrations that might be observed in untreated wastewater samples are not likely to inhibit the biologic treatment in an activated sludge treatment plant. Similarly, the concentrations in receiving water are usually too low to observe an acute mortality on marine bacteria. The freshwater algae are 90

times more sensitive than the bacteria and the concentration that might be encountered in receiving water might pose a threat to algal communities. Photodegradation slightly affect the toxicity of TCS to algae however different from the EC_{50} values, the EC_{10} value for the photolyzed TCS is smaller than the unphotolyzed TCS. This result points out the importance of the effect of photolysis on the toxicity of TCS. Nevertheless, other possible effects of TCS including endocrine disrupting effects and creating antibacterial resistance cannot be ruled out and should be studied.

İKİ FARKLI SAF KÜLTÜR VE AKTİF ÇAMUR ÜZERİNDE TRIKLOSAN TOKSİSİTESİNİN BELİRLENMESİ

ÖZET

Kişisel bakım ürünlerinin ve farmasötik kullanımının artmasıyla birlikte bu maddelerin çevreye etkileri giderek daha fazla dikkat çekmektedir. Bir çok olası etkisi bulunabilen ve sucul ortamda çeşitli organik ve inorganik maddelerle birlikte rastlanan bu tür maddelerin toksik etkileri araştırılmaktadır. Bu deneysel çalışmalar genellikle hem hızlı çoğalmaları hem de kısa yaşam döngülerine sahip olmaları nedeniyle tek hücreli organizmalar üzerinde yürütülmektedir. Tek hücrelilerde görülen etki yüksek yapılı canlılara olabilecek toksik etkilerin bir göstergesi kabul edilebilir.

Triklosan (TCS) 1960'ların başında sentezlenen geniş spektrumlu antimikrobiyal bir ajandır. Triklosan poliklorinlenmiş aromatikler grubuna dahil olup kimyasal yapısı 5-kloro-2-(2,4-diklorofenoksi) fenol şeklindedir. Gram (+) ve Gram (-) bakteriler üzerinde etkili olduğu kadar bazı maya ve küf mantarı türleri üzerinde de etkilidir. Günümüzde kullanımı hızla yaygınlaşarak artan anti-bakteriyel sabunlarda, diş macunlarında, deodorantlarda, kozmetik ürünlerinde kullanılabildiği gibi yiyecek ambalajlarında ve bazı özelleşmiş kumaşlarda da kullanılmaktadır. Kişisel bakım ürünlerinde, ürün ağırlığının genellikle %0.1 - 0.3'ü oranında bulunan TCS'nin küresel toplam kullanım miktarı 1500 ton/yıl'ı geçmektedir. Bazı ülkelerce kullanımı diğer antimikrobiyal maddeler gibi kısıtlandırılmış olsa da küresel olarak uygulanabilecek bir kısıtlamadan söz etmek mümkün değildir.

TCS insanlar tarafından farklı amaçlar için kullanıldıktan sonra %96 oranında atıksuya karışmaktadır. Her ne kadar antimikrobiyal bir bileşik de olsa aktif çamur prosesiyle %96'ya kadar giderilebilmektedir. Ancak TCS'nin giderilemeyen kısmı atıksu arıtma tesislerinden alıcı ortama deşarj edilmektedir. TCS yüzey sularında genellikle 0.5-30 μg/L aralığında tespit edilmiş olsa da arıtılmamış atıksularda hastane atıksuları gibi atıksularda 0.56 mg/L gibi yüksek konsantrasyonlar bazı çalışmalarda belirtilmiştir. TCS'nin antimikrobiyel bir madde olması aktif çamurda bulunan karışık kültür üzerinde bir inhibisyon etkisi ihtimalini akla getirmektedir. Benzer şekilde yüzey sularında tespit eden konsantrasyonlar da algler gibi görece hassas türler üzerinde toksik etki gösterebileceğini düşündürmektedir. Bu nedenle bu tez çalışmasında TCS'nin olası toksik etkilerinin incelenmesi amaçlanmıştır.

Çalışmada TCS'nin olası toksik etkileri hem tatlı su hem de tuzlu su alıcı ortamı için ayrı ayrı incelenmiştir. Tatlı su ortamındaki toksik etkilerin belirlenebilmesi için tek hücreli bir tatlı su algi olan *Pseudokirchneriella subcapitata* tuzlu su ortamındaki etkilerin belirlenebilmesi içinse biyolüminesans özelliğe sahip bir denizel bakteri olan *Vibrio fischeri* saf kültürleri seçilmiştir. Deniz suyunda yaşayan bir alg türü olan *Phaeodactylum tricornutum* üzerinde de farklı TCS konsantrasyonlarıyla deneysel

çalışmalar yürütülmüştür. TCS'nin biyolojik arıtma tesisleri üzerinde olası toksik etkisini belirlemek için ise aktif çamur karışık kültürü üzerinde oksijen tüketim hızı (OTH) deneyleri giriş atık suyunda bulunan farklı TCS konsantrasyonlarıyla yürütülmüştür.

OTH deneylerinde öncelikle literatürde belirtilmiş olan yüksek konsantrasyonlara yakın olan 0.8 mg/L deney konsantrasyonu olarak seçilmiştir. Bu konsantrasyonda herhangi bir inhibisyon etkisi görülmediği için deneyler 1.6 mg/L ve 6 mg/L'lik TCS konsantrasyonları ile tekrarlanmıştır. 1.6 mg/L TCS konsantrasyonunda da herhangi bir inhibisyon etkisi görülmezken, 6 mg/L deney konsantrasyonunun, μH ve Ks üzerindeki etkiyle karakterize edilen yarışmasız inhibisyona (un-competitive) yol açtığı gözlemlenmiştir. Literatürde daha yüksek konsantrasyonlardaki çalışmaları bulmak mümkün olsa da bu çalışmalar TCS'nin sudaki çözünürlüğünü aşan konsantrasyonlarla, taşıyıcı bir solvent (methanol, aseton vb.) kullanılarak yürütülmüş olduğu ve gerçek durumda bu konsantrasyonlarla atıksuda karşılaşılamayacağı göz önünde bulundurulmalıdır. Bütün bu verilerin ışığında TCS'nin ekstrem durumlar dışında aktif çamur sistemleri üzerinde olumsuz bir etkisi olamayacağı görülmüştür.

Tuzlu sudaki toksik etkinin gözlenebilmesi için *V. fischeri* üzerindeki deneyler biyolüminesans inhibisyonunu belirlemek amacıyla ekotoksikolojik deneylerde uzun yıllardır kullanılan ve hızlı sonuç veren Microtox® protokolü ile yürütülmüştür. TCS nin *V. fischeri* üzerindeki toksik etkisinin göstergesi olarak EC_{50} değeri %95 güven aralığında 0.67 mg/L olarak saptanmıştır. Bu değer 72 saatlik üreme inhibisyonu testiyle (EN ISO 8692:2004) *P. subcapitata* için 7.74 μg/L bulunmuştur. Benzer protokol bazı modifikasyonlarla *P. tricornutum* üzerinde de uygulanmış ancak 0.4 ve 4 μg/L TCS konsantrasyonlarında herhangi bir üreme inhibisyonu görülmemiştir. EC_{50} değerlerine göre *P. subcapitata*'nın *V. fischeri*'ye göre 90 kat daha hassas olduğu görülmüştür. TCS'nin alg populasyonu üzerindeki etkisi üzerinde daha geniş bir kanıya varabilmek için EC_{10} değeri olarak da tanımlanabilecek en düşük etki gözlemlenen konsantrasyon (LOEC) değerleri analiz edilmiş ve EC_{10} değeri 3.5 μg/L olarak bulunmuştur. TCS yüzey sularında genellikle 0.05 ila 2.3 μg/L aralığında bulunduğundan her ne kadar EC_{50} değerine yakın olmasa da LOEC değerlerine yakın bulunması TCS'nin alg türleri üzerinde toksik etki gösterebileceği açısından anlamlıdır.

Diğer poliklorofenoller gibi TCS de doğal güneş ışığına veya yapay UV ışınına maruz bırakıldığında parçalanmaya uğramaktadır. Bu nedenle TCS'nin çevredeki akıbeti üzerinde etkili olabilecek önemli faktörlerden biri olan doğrudan ve dolaylı fotoliz reaksiyonunun TCS'nin toksik etkisini ne yönde etkileyeceğinin belirlenmesi için 3 saat UV ışınına maruz bırakılan TCS kullanılarak *V. fischeri* ve *P. subcapitata* ile toksisite deneyleri tekrar edilmiştir. Doğrudan fotoliz sonucunda *V. fischeri* için EC_{50} değeri 1.29 mg/L'ye yükselmiştir. LC-MS/MS ile yapılan ölçüm sonucuna göre 3 saat UV ışınına maruz bırakılan TCS'nin %40 oranında parçalandığı tespit edilmiş ve EC_{50} değerindeki yükselmenin bu parçalanmaya bağlı olduğu doğrulanmıştır. Buna ek olarak literatürdeki çalışmalarda TCS'nin fotoliz ürünü olarak 2,4 DCP (m/z: 189) oluşumu iddia edilse de LC-MS/MS analizlerinde bu iyona rastlanmamıştır. TCS doğal ortamdaki yüzey sularında çözünmüş organik bileşiklerle birlikte bulunması beklendiğinden hümik madde (5 mg/L TOC eşdeğeri) içeren TCS çözeltisi ile dolaylı fotoliz deneyleri *V. fischeri* için tekrarlanmıştır. Her ne kadar de LC-MS/MS

analizlerinde foto-parçalanma doğrudan fotolize göre biraz daha fazla gerçekleşmiş olsa da, *V. fischeri* için EC_{50} değeri ancak 1.37 mg/L'ye yükselmiştir. Dolaylı fotolizle yaklaşık olarak %64 TCS giderimi olmuş olsa da bu giderim EC_{50} değerinde ancak %51'lik bir değişime yol açmıştır. Bu sonuç dolaylı fotoliz ile ortaya çıkan yan ürünlerin de toksik etkisi bulunabileceğini göstermektedir. Doğrudan fotolizin, TCS'nin *P. subcapitata* toksisitesi üzerindeki etkisi *V. fischeri* ile karşılaştırıldığında daha düşük olmuştur. *P. subcapitata* için fotoliz sonrası EC_{50} değeri 8.70 μg/L olarak bulunmuştur. EC_{50} değeri fotoliz sonrasında yükselirken EC_{10} değeri ise 3.5 μg/L den 0.6 μg/L düşmüştür. Benzer şekilde, EC_{25} değeri de 5.96 μg/L den 4.60 μg/L'ye düşmüştür. EC_{10} ve EC_{25} değerlerindeki bu düşüş TCS'nin fotoliz ürünlerinin de alg populasyonu üzerinde toksik etki gösterebileceğine işaret eder ve TCS yüzeysel sularda bu değerlere yakın konsantrasyonlarında bulunabileceği için dikkat çekici bir sonuçtur.

TCS'nin antimikrobiyal bir madde olmasından dolayı ilk akla gelen çevresel etki, maddenin akut toksik etkisi olsa da, gerçek sistemlerde gözlenebilecek etkileri daha farklı olabilir. Örneğin, TCS'nin bu çalışmada olduğu gibi saf su içinde bulunmayıp doğal ortamdaki sularda başka antimikrobiyaller, antibiyotikler ve farklı kişisel bakım ürünleriyle birlikte bulunması, sinerjistik bir etkiye yol açarak beklenenden daha fazla toksik etki göstermesine neden olabilir. Ayrıca, bu konu hakkında literatürde çok az sayıda çalışma olsa da TCS, kabuklular ve yumuşakçalar gibi yüksek yapılı canlılarda biyobirikime sebep olabilir. Bir başka önemli konu da TCS'nin antimikrobiyal madde oluşundan ve TCS içeren kişisel bakım ürünlerinin günlük yaşantıda büyük miktarlarda kullanılmasından dolayı bakteri direnci oluşumuna yol açabilme olasılığıdır. Bazı çalışmalarca TCS'nin bakteri direnci göstermediği sonucuna varılsa da çalışmalar arasında tam bir uzlaşma bulunmamaktadır. TCS'nin yine başka antibiyotiklerle birlikte karışık kültür ortamlarında bulunduğu durumlar için çapraz bakteri direncine yol açma ihtimali ayrıca incelenmelidir. TCS'nin bir diğer olası etkisi de, hormonal sistemler üzerindeki etkidir. TCS'nin yapısal olarak tiroid hormanlarına benzer oluşu ve bazı büyüme ve seks hormanları üzerindeki etkileriyle ilgili çalışmalar da henüz tam bir uzlaşmaya erişememiş olsa da bazı çalışmalarda hermafrodit organizmalar üzerinde ciddi etkileri gözlenmiştir. TCS'nin mayalar üzerindeki östrojenik etkisi de ileride incelenmesi gereken önemli bir konudur.

1. INTRODUCTION

The presence of pharmaceuticals and personal care products (PPCP) in the environment is of growing concern. Triclosan (TCS) is an antimicrobial agent that is widely used in soaps, toothpaste, mouthwash, fabric, deodorant and cosmetics as well as in textiles. Concentrations of TCS in PCPP's are typically 0,1-0,3%. TCS is listed on the Organisation for Economic Cooperation and Development's (OECD) High Production Volume chemicals list (OECD, 2004 - Url-1). For North America, Europe and Asia total consumption of TCS exceeds 1500 ton per year and this amount rises year to year. As an antimicrobial agent TCS is effective against gram (+) and gram (-) bacteria, yeasts and molds by inhibiting the fatty acid synthesis but has no effect on bacterial spores.

Nearly 96% of TCS rinsed down to the sewer system and discharged with wastewater and has been commonly detected in wastewater and surface water samples ranging from a few μg/L to several hundreds μg/L. TCS containing wastewater can be treated effectively (up to 95%) by activated sludge process but a small amount of the compound discharged with the wastewater treatment plant effluent or illegally discharged to the receiving environments. Because of being an antimicrobial agent TCS is expected to have a toxic effect on aquatic organisms and an inhibitory effect on activated sludge biomass. Moreover, it has been reported that TCS may cause bacterial resistance and may act as an endocrine disruptor because of the structural similarity to the anthropogenic estrogens and thyroid hormones. Nevertheless, the main observed effect of TCS is acute toxicity on unicellular microorganisms. Some bio-accumulative effects on vary aquatic organisms are observed dependent on the species of the organisms (Coogan & La Point, 2008).

There are some concerns about photolytic degradation of TCS for a decade. The photodegradation products of TCS are reported as 2,8-dichlorobenzodioxin and its

other dioxin derivatives due to exposure to the UV light. These photoproducts may also have toxic effects on aquatic system and activated sludge biomass.

1.1 Purpose of Thesis

The aim of the thesis is to determine the possible adverse effects of TCS on aquatic organisms both during the treatment of wastewater that contains TCS and after the discharge of treated wastewater effluent to receiving environments.

To investigate toxic effects of TCS on aquatic organisms ecotoxicological experiments were conducted. Selected pure cultures are a marine bacteria, *V. fischeri*, a marine algae *P. tricornutum* and a freshwater algae, *P. subcapitata*. EC_{50} values are found by luminescence inhibition experiments for the *V. fischeri* and algal growth inhibition tests fort the *P. subcapitata*. In this way toxic effects of TCS is determined both in seawater and freshwater. Experiments are repeated after exposing the aqueous solution of TCS for a predetermined period to the UV light to demonstrate the effects of photodegradation by-products of TCS on aquatic organisms. Moreover, the acute toxic effects of TCS on activated sludge biomass is investigated by conducting oxygen utilization rate experiments to estimate the possible operational problems in activated sludge treatment system due to TCS in the wastewater influent.

2. LITERATURE REVIEW

2.1 General Properties of Triclosan

2.1.1 Chemical properties of TCS

Triclosan was originally developed by Ciba-Geigy Company, Basel, Switzerland, in the early 1960s. (Jones et al., 2000). In the period from 1992 to 1999, a majority of the 700 antibacterial products on the market contained TCS as an active ingredient (Schweizer, 2001) Triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol], a halogenated phenol, is a non-ionic broad spectrum antimicrobial compound. It is also poorly soluble in water (10 mgL^{-1} at 20 $^{\circ}C$) and has an estimated log octanol - water partition coefficient ($LogK_{ow}$) of 4.8 at neutral pH (Price et al., 2010) and, a pKa of 7.9 to 8.1, and a vapor pressure of (Orvos et al., 2002) TCS may be classified as a halogenated aromatic hydrocarbon, containing phenol, diphenyl ether and polychlorinated biphenyl functional groups (Ahn et al., 2008).

TCS is a common name of commercial products as known as IrgasanDP300, Aquasept, Sapoderm and Ster-Zac. Fibres and other materials that have TCS incorporated into them may be referred to as Ultra-Fresh, Amicor, Microban, Monolith, Bactonix and Sanitized (Erici et al., 2002).

Triclosan is produced by treatment of [2,4,4'-trichloro-2'-methoxydiphenyl ether] with aluminium chloride in benzene under reflux. Under extreme conditions such as high alkalinity and heat, conversion to chlorinated dibenzo-p-dioxins can occur. (Fiege et al., 2000). The type and purity of the starting materials in the synthesis of triclosan will influence the extent of contamination by the impurities dioxins and dibenzofurans. (Ciba Specialty Chemicals, 2001). Commercial grades of triclosan are typically over 99% pure. Impurities that may be present in trace amounts are shown in Table 2.1.

Table 2.1: Impurities of commercial Triclosan(SCCP/1192/08, 2009)

Individual related-compound (Gas Chromatography)	$\leq 0.1\%$
Total related compounds	$\leq 0.5\%$
2,4 Dichlorophenol	$\leq$ 10mg/kg
Sum of 3 and 4 Chlorophenol	$\leq$ 10mg/kg
2,3,7,8 Tetrachlorodibenzo-p-dioxin	≤ 0.001 μg/kg
2,3,7,8 Tetrachlorodibenzo-furan	≤ 0.001 μg/kg
2,8 - Dichlorobenzo-furan	$\leq$ 0.25 mg/kg
2,4,8 - Trichlorodibenzo-furan	$\leq$ 0.5 mg/kg
Ash	$\leq$ 0.5 mg/kg
Mercury	$\leq$ 1 mg/kg
Arsenic	$\leq$ 2 mg/kg
Antimony	$\leq$ 10 mg/kg
Lead	$\leq$ 10 mg/kg
Cadmium	$\leq$ 5 mg/kg
Nickel	$\leq$ 10 mg/kg
Copper	$\leq$ 10 mg/kg
Chromium	$\leq$ 2 mg/kg
Sum of heavy metals as lead sulfide precipitation	$\leq$ 20 mg/kg

Table 2.2: Chemical identity of Triclosan (NICNAS, 2009 - Url-2)

Property	Value, name or structure
CAS No :	3380 - 34 - 5
Synonyms :	Triclosan 2,4,4' - trichoro - 2' - hydroxydiphenyl ether; Ether, 2'-hydroxy-2,4,4'-trichlorodipehenyl; Phenyl ether, 2'hydroxy-2,4,4'- tricholoro-; 2',4',4-Trichloro-2-hydroxydiphenyl ether; 2',4,4-Trichloro-2-hydroxydiphenyl ether; 2'-Hydroxy-2,4,4'-tricholorodiphenyl ether 2,2'-Oxybis(1'.5'-dichlorophenyl-5-chlorophenol) 2-Hydroxy-2',4,4'-tricholorodiphenyl ether; 3-Chloro-6-(2,4-dichlorophenoxy) phenol; 4-Chloro-2-hydroxyphenyl 2,4-dichlorophenyl ether
Trade names :	CH3565; Bacti-Stat soap; DP 300; Irgacare MP; Irgacide LP 10; Irgaguard B 1000; Irgasan, Irgasan CH 3565; Irgasan DP 30; Irgasan DP 300; Irgasan DP 3000; Irgasan PE 30; Irgasan PE 30; Irgasan PG 60; Microban Additive B; Microban B; NM 100; TCCP; THDP; Tinosan AM 100; Tinosan AM 110; Tinosan NW 500; Tinosan CEL Liquid; Ultrafresh NM 100; Vinyzene DP 700; Yujieksin; Zilesan UW
Molecular Formula	$C_{12}H_7C_{13}O_2$
Molecular Weight	289.54 g/mol

Table 2.3: General physical properties of TCS

Property	Value	Reference
Melting Point	54 57°C	Ganem et al.,2011
Decomposition Temperature	Above 200°C	Ganem et al.,2011
Specific gravity	1.55 × 103 kg/m^3 at 22 °C	EPA, 2007 - Url-3
Solubility		
Distilled Water 20°C	0.001g/100g (1 ×10^{-5} g/mL) at 20°C	Ciba Specialty Chemicals (2001)
Distilled Water 50°C	0.004g/100g (1 ×10^{-5} g/mL) at 20°C	
n-hexane	8.5 g/100g (0.085 g/mL) at 25°C	
Ammonium hydroxide	0.30 g/100g (0.003 g/mL) at 25°C	
Acetone	≤ 100g/100g (≤ 1.0 g/mL) at 25°C	
Fat	≤ 65g/100g at 37°C	EPA, 2007
Methanol	0.7g/100g (7 × 10^{-3} g/mL) at 20°C	Lavecchia & Zuroro,2008
Ethanol	≤ 100gTCS/100g	SCCP/1192/08, 2009 - Url-4
Acetone	≤ 100gTCS/100g	SCCP/1192/08, 2009 - Url-4
1 N caustic soda 31.7	31.7g/100g	SCCP/1192/08, 2009 - Url-4

2.1.2 Physical properties of TCS

Triclosan has a solid, white powder structure in room temperature and has a specific odour. General physical properties of TCS is given by the table 2.3.

2.1.3 Use of TCS

The first US patent for triclosan was in 1964 (Merck, 1983) and it has been used in wide ranged products such as:

- soaps
- hand-washes
- dish-washing products
- laundry detergents and softeners
- plastics (e.g., toys, cutting boards, kitchen utensils)
- toothpaste and mouth washes
- deodorants and antiperspirants
- cosmetics and shaving creams
- acne treatment products

- hair conditioners
- bedding
- trash bags
- apparel like socks and undershirts
- hot tubs, plastic lawn furniture
- impregnated sponges
- surgical scrubs
- implantable medical devices
- pesticides (APUA, 2011 - Url-5)

These products contain TCS at several levels, for example personal care products are the most common form antimicrobial which are contain TCS at concentrations of 0.1-0.3%. Levels contained by the products regulated by the US Food and Drug Agency (USFDA) in United States and European Community Cosmetic Directive in European Union (Rodricks et al., 2010). In EU Triclosan maximum allowed level of Triclosan is 0.3% in cosmetic products like EU and in Canada TCS's maximum allowable levels are 0.03% for mouthwashes and 0.3% for other cosmetics. In Japan maximum allowable concentration is much more restricted than EU, for cosmetic products, 0.1% (Ministry of Healthand Welfare Notification No. 331, 2000). In addition to this restriction there are also some regulations about impurities of TCS. For Canada the concentration of the impurities 2,3,7,8-tetra-chlorodibenzo-p-dioxin and 2,3,7,8-tetra chloro dibenzofuran must not exceed 0.1 ng/g, the total concentration of all other chlorinated dibenzodioxin and dibenzofuran impurities must not be greater than 10 μg/g and no other individual impurity should be greater than 5 according to regulations (Health Canada, 2005). Similar to Canada, in the United States, because of the formation of dioxins and dibenzofurans as by-products impurities of TCS, limited for the following compounds: less than 10 μg/g for monochlorophenols; less than 10 μg/g for 2,4-dichlorophenol; less than 0.25 μg/g for 1,3,7-trichlorodibenzo-p-dioxin; less than 0.5 μg/g for 2,8-dichlorodibenzo-p-dioxin; less than 0.25 μg/g for 2,8-

dichlorodibenzofuran; less than 0.5 μg/g for 2,4,8 trichlorodibenzofuran; less than 1 pg/g for 2,3,7,8-tetrachlorodibenzo-p-dioxin; and less than 1 pg/g for 2,3,7,8-tetra chlorodibenzofuran by the United States Pharmacopoeia (USP) (USP, 2004 - Url-6) However detailed data can be obtained from Europe, Canada and USA except Australia that is still preparing a national perspective for using of the TCS, no data could be obtained from Asian, African and South American countries.

Globally, TCS use was increased between 1992 to 1999 then production of TCS relatively stabilized in recent years. According to study of Adolfsson-Erici et. al. (2002) in Sweden there is 2 tons of TCS consumption per year, this consumption value is approximately 40 tons per year for Germany (Xie et al.,2008). Overall quantity used within the EU reached approximately 450 tons in the year 2006. (SCCP/1192/08, 2009). In USA total consumption of TCS is more than 300 tons per year (Halden and Paull, 2005). Total consumption of TCS in the world has exceeded 1500 tons per (Singer et al., 2002).

These TCS consumption data for consumers daily life suggest that TCS can be detected in wastewater samples and even in treated wastewater effluents and a study of its toxic effects in aqueous environment is necessary.

2.1.4 Mechanisms of action

Triclosan's antibacterial action involves the lipid synthesis. It acts as an inhibitor of the enoyl-acyl reductase (ENR) enzyme which is encoded by the gene FabI responsible for the lipid synthesis in the cell by mimicking its natural substrate. ENR catalyses the final, regulatory step in the fatty-acid synthase cycle: the reduction of a carbon carbon double bond in an enoyl moiety that is covalently linked to an acyl carrier protein.

This binding action increases the enzyme's affinity for nicotinamide adenine dinucleotide (NAD^+). This results in occurring of the ENR-NAD^+- Triclosan that is not able to participate in fatty acid synthesis. It also permeates to the cell wall and affects inner cytoplasmic membrane sites. By these actions also prevents bacterial growth by affecting formation of cell walls (Levy et al., 1999; McMurry et al., 1998; Heath et al., 1999).

In concentrations greater than 0.01~0.1 Triclosan induces K^+ leakage showing membrane damage. TCS also demonstrates Z-pattern type of adsorption which is responsible for the destruction of membrane and the generation of new adsorbing sites (Russell, 2004).

2.2 Bacterial Resistance and Cross Resistance

After manufacturing and widely using of TCS for over 40 years in PCPP -like hand soaps- questions were raised about causing of antimicrobial resistance by TCS. Studies about this concern started in the beginning of 1990's. It was investigated the effect of 0.15% TCS containing deodorants on the normal bacterial community but no resistant bacterial populations were detected and also no opportunistic species were developed during the 6 months of test period (Cox, 1987). In addition two independent double-blind studies conducted and proved that, continuous contact for 1 year period of dentifrice containing 0.3% TCS did not cause any resistant oral microorganisms and opportunistic microorganisms (Volpe et al., 1993). Supporting this result 0.2% TCS containing dentifrice for 7 months of contact period did not developed resistance(Jones et al., 2000). On the contrary it is suggested that TCS has the same mechanisms of action like some kind of antibiotics thus widespread use of TCS may help the microbial resistance as cross-resistance to antibiotics hence experimental studies on pure cultures of methicillin resistant Staphylococcus aureus (MRSA) conducted with maximum inhibitory concentrations (MICs) of TCS (ranged between 0.025 mg/L to 1 mg/L) Continuous exposure of sub-MIC concentrations of TCS for 1 month did not changed effectiveness of TCS on MRSA (Suller and Russel, 2000; Schiweizer, 2001). Thus this concern did not proven yet by the experimental studies (Russel, 2003; Aiello, 2006).

In summary all laboratory studies were sustained on pure cultures and isolated environments so studies have to conducted under much more realistic conditions and interaction of TCS with different antibiotic compounds.

2.3 Occurrence of TCS in Aquatic Environment

Nearly 96% of the PPCP products which contain TCS discharged with domestic and industrial wastewater. Seasonal changes and demographics of the regions may effects the occurrence of TCS in the wastewaters and surface water. However studies are scarce about TCS from predeveloped countries or third-world countries, it could be concluded that industrialized and developed countries have higher levels of TCS both in wastewater and surface water.

Although recent studies demonstrated that TCS can be removed highly efficient (up to 98%) by the activated sludge process 100% removal rate can not be reached. Thus 5-10% of TCS could be found in surface water and TCS concentrations should be monitored due to its toxic effects on aquatic species.

Table 2.4: Worldwide concentrations of TCS found in WWTP influents

WWTP Location	**Min. Concentration of TCS (μg/l)**	**Max. Concentration of TCS (μg/l)**	**Mean ($\pm$ sd) (μg/l)**	**Reference**
USA	0.8	10.8	4.7 ± 1.6	Heidler & Halden (2007)
Germany	1	1.2	1.2 ± 0.08	Bester (2003)
USA	3.8	16.6	-	McAvoy et al. (2002)
Germany	0.372	0.841	-	Wick et al. (2010)
Greece	0.5	30	-	Stasinakis et al. (2008)
Spain	0.39	4.2	1.8	Gomez et al. (2007)
Japan	2.7	11.9	-	Nakada et al. (2010)
Canada	0.01	4.01	-	Lishman et al. (2006)
Japan	0.055	0.134	-	Nishi et al. (2008)
USA	6.74	10	-	Jackson & Sutton (2008)
USA	1.86	26.8	-	Chalew & Halden (2009)
Wales	-	-	0.38	Miege et al. (2009)
India	0.142	0.213	-	Kettrup & Cai (2007)
Greece	-	-	0.445	Pothitou and Voutsa (2008)
UK	33	463	-	Hordern et al. (2008)
USA	0.181	0.608	0.046	Yu and Chu (2009)
Spain	-	-	0.046	Rodil et al. (2009)
Spain	1.3	37.8	-	Agüera et al. (2003)
USA	-	-	0.488	Richard et al. (2010)
Sweden	-	-	0.38	Bendz et al. (2005)
Australia	0.573	0.845	-	Ying and Kookana (2007)
Spain	-	-	0.86	Rosal et al(2010)
USA	0.181	0.608	-	Yu and Chu (2009)
Germany	0.372	0.841	-	Wick et al.(2010)
Spain	-	-	0.046	Rodil et al. (2009)
Switzerland	0.5	1.3	-	Lindström et al.(2010)
Spain	-	560		Mezcua et al. (2004)

Table 2.5: Worldwide concentrations of TCS found in WWTP effluents

WWTP Location	**Min. Concentration of TCS (μg/l)**	**Max. Concentration of TCS (μg/l)**	**Mean ($\pm$ sd) (μg/l)**	**Reference**
USA	0.01	10.08	0.07 ± 0.06	Heidler and Halden (2007)
Germany	0.043	0.059	0.057 ± 0.077	Bester (2003)
USA	0.24	2.7	-	Mc Avoy (2002)
Germany	0.011	0.482	0.14	Price et al.(2010)
Spain	0.022	0.087	-	Pedrouzo et al.(2009)
India	-	-	0.022	Kettrup and Cai (2007)
Germany	0.0125	0.162	-	Wick et al (2010)
USA	0.42	0.9	-	Jackson and Sutton (2008)
Spain	0.04	0.8	0.2	Gomez et al.(2007)
Greece			0.076	Pothitou and Voutsa (2008)
China	-	-	0.071	Zhao et al (2010)
Canada	0.01	0.324	-	Lishman et al(2006)
UK	0.013	0.082	-	Hordern et al (2008)
Spain	-	-	0.045	Kuster et al(2008)
USA	-	-	0.072	Yu and Chu (2009)
Spain	-	-	0.019	Rodil et al (2009)
USA	0.010	0.029	-	Boyd et al (2003)
Australia	0.023	0.434	0.108	Ying and Kookana (2007)
Spain	0.4	22.1	-	Agüera et al (2003)
Spain	-	-	0.219	Rosal et al (2010)
Switzerland	0.1	0.65	-	Lindström et al.(2010)

Table 2.5 Worldwide concentrations of TCS found in WWTP effluents (continued)

WWTP Location	**Min. Concentration of TCS (μg/l)**	**Max. Concentration of TCS (μg/l)**	**Mean ($\pm$ sd) (μg/l)**	**Reference**
USA			0.071	Richard et al (2010)
Switzerland	0.011	0.098	-	Singer et al. (2002)
USA	0.2	2.7	-	McAvoy et al. (2002)
USA	-	-	0.07$\pm$0.06	Heidler and Halden (2007)
USA	0.03	2.7	-	Halden and Paull (2005)
USA	-	-	0.19	Fair et al. (2009)
Canada	0.01	0.324	-	Lishman et al. (2006)
Sweden	-	-	0.16	Bendz et al. (2005)
Japan	0.03	2.7	-	Nakada et al. (2010)
Spain	-	-	0.219	Rosal et al(2010)

Table 2.6: Worldwide concentrations of TCS found in surface water samples

Sample Location	**Min. Concentration of TCS (μg/l)**	**Max. Concentration of TCS (μg/l)**	**Mean ($\pm$ sd) (μg/l)**	**Reference**
USA	0.05	0.085	-	Price et al.(2010)
USA	-	-	0.056	Loraine and Pettigrove (2006)
Switzerland	-	-	0.074	Lindstrom et al. (2002)
India	0.031	0.037	-	Kettrup and Cai (2007)
Germany	0.03	0.268	-	Wick et al.(2010)
China	-	-	0.0145	pen. (2010)
China	0.048	1.023	-	Peng et al.(2008)
China	0.0013	0.242	-	Zhao et al.(2010)
UK	0.005	0.024	-	Hordern et al (2008)
UK	0.019	0.08	-	Sabaliunas et al(2003)
Romania	-	-	0.0382 ± 0.0056	Moldovan (2006)
Spain	-	-	0.0813	Rosal et al. (2010)
USA	ND	2.3	-	Kolpin et al. (2002)
USA	0.43	0.104	-	Morrall et al. (2004)
Germany	0.0003	0.01	-	Bester (2005)
Australia	ND	0.075	-	Ying et al. (2007)
Japan	0.0006	0.0591	-	Nakada et al. (2008)
Swiss	0.011	0.098	-	Singer et al. (2002)
USA	-	-	1.6	Halden and Paull (2005)
USA	0.0049	0.014	0.0075	Fair et al. (2009)
Australia	0.045	0.187		Ying and Kookana (2007)
Spain	-	-	0.045	Kuster et al.(2008)
Switzerland	0.002	0.014	-	Lindstorm et al. (2002)

2.4 Toxicity of TCS

Toxicity can be determined as potential adverse effects of a substance to living organisms. It can be measured by its effects on the target organism. Population - level measure of toxicity is often used which relates the probabilities of an outcome for a given individual in a population. This basic principle is referred as the concentration-response relationship. The responses of test organisms to the different chemical concentrations yield a characteristic S-shaped (sigmoid) curve. The least variability is at the 50% level of response. In determining effects other than mortality EC_{50} (median effective concentration) is usually used. Generally EC_x values can be defined as a point estimate of the toxicant concentration that would cause a given percentage of reduction in a sub-lethal biological measurement such as reproduction, bioluminescent and growth. The no-observed-effect concentration (NOEC) is defined as the highest tested toxic compound concentration that results in no statistically significant difference from a control and the lowest observed effect concentration (LOEC) is defined as the lowest has a statistically significant difference from the control. For more thorough analysis different EC_x values could be used such as EC_{10}, EC_{25} and EC_{80} by investigator customarily.

2.4.1 Toxicity of TCS on microorganisms

TCS is a water-borne pollutant thus experimental studies about acute toxicity generally focused on the aquatic organisms, mostly on unicellular organisms because of their high growth rates and short life cycles.

In different studies are based on algal growth inhibition test (OECD guideline 201) is usually used. Effect of TCS on growth of *Scenedesmus subspicatus* which is a freshwater algae was observed that at 0.6 μg/l. Higher inhibition of *S. subspicatus* observed as the concentration of TCS increased at 72^{nd} hour. For another freshwater algae *Anabena flos-aquae* the 96-h EC_{10} and EC_{90} values were found as 0.97 μg/l and 5.6 μg/l and NOEC was found 0.81 μg/l (Orvos et al., 2002).

Toxicity of TCS to *P. subcapitata* (*S. capricornutum*) examined by various studies that using the algal growth inhibition tests. EC_{25} and EC_{50} reported as 2.44 μg/l and 4.88

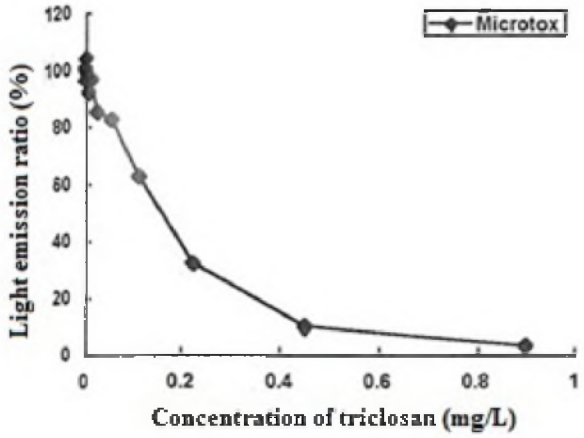

Figure 2.1: Effects of TCS on *Vibrio fischeri* (Tatarazako et al., 2004)

μg/l relatively by Orvos et al (2002). Supporting that results Tatarazako et al (2004) reported IC_{25} and IC_{50} values 3.4 μg/L and 4.7 μg/L relatively. Differently EC_{50} value for *P. subcapitata* nearly ten times smaller than other results found and reported as 0.53 μg/L.

In addition studies were also conducted on different marine species. EC_{25} and EC_{50} for *Skeletonema costatum* which is a marine diatom are found higher than 66 μg/L showing more endurance against TCS than freshwater algae (Orvos et al., 2002). *Dunaliella tertiolecta* is another marine algae species that tested for the toxicity of TCS. The 96-hour EC_{50} value for *Dunaliella tertiolecta* reported as 3.55 μg/l (DeLorenzo & Fleming, 2008).

Moreover toxicity studies can be found on marine bacteria. *V. fischeri* is a bioluminescent bacteria which used as a key for toxicity experiments. Toxicity experiments on *V. fischeri* usually made with Microtox® acute toxicity protocol. The IC_{25} and IC_{50} value reported as 0.07 mg/L and 0.15 mg/L after 15 minutes of exposure time (Tatarazako et al.,2004). Effect of TCS on bioluminescent ratio of *V. fischeri* shown in Figure 2.1. Supporting that EC_{50} values reported as 0.28 mg/L (Farre et al., 2008) and 0.22 mg/L (Stasinakis et al., 2008) by different studies using the same method.

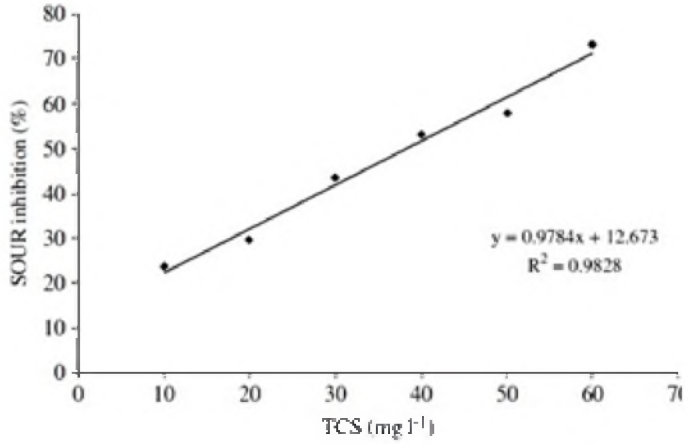

Figure 2.2: Effects of TCS on specific (SOUR inhibition θ_c= 5 days (Stasinakis et al., 2008)

2.5 Toxicity of Triclosan on Activated Sludge Microorganisms

Due to antibacterial effects of TCS concerns was raised about affects on activated sludge biomass. Differently from isolated pure cultures toxicity tests on mixed cultures are conducted by specific oxygen utilization rate (OUR) tests. 3 hour median inhibitory concentration reported as 20 mg/L by using SOUR test with 0, 1.0, 3.2, 10, 32, and 100 mg/L initial TCS concentrations in a batch system. 0.5% and 1.4% inhibition also observed on glucose uptake rate for TCS concentrations of 3 and 10 mg /l respectively (Orvos et al.,2002). On the other hand for continuous activated sludge (CAS) systems different sludge ages may changes inhibitory effect of TCS. EC_{50} values reported 38.2 and 9.97 mg/L for 5 to 15 days of sludge ages by using test concentrations of TCS 0.05 to 50 mg/L. Increased TCS toxicity on SOUR may be attached to the formation of smaller flocs obtained at higher sludge ages. As an example inhibitory effect of TCS on SOUR shown in the Figure 2.2.

In addition results on biological oxygen demand (BOD) removal rate constant can be found from the literature. EC_{50} value for the BOD removal rate constant published as 1.82$\pm$1 mg/L for 2 mg/L TCS by the standart 5 day BOD test (Neumegen et al., 2004).

More detailed inhibitory concentrations for various species and activated sludge microorganism can be found in the Table 2.7 below:

Table 2.7: Effects of TCS on freshwater, seawater and activated sludge microorganisms

Test Species	Test Media	Test Period	EC_{50}	Reference
S. subspicatus (Green algae)	Freshwater	96-h	1.4 μg/L	Orvos et al. (2002)
S. capricornutum (P. subcapitata,Green algae)	Freshwater	96-h	4.7 μg/L	Tatarazako et al. (2004)
S. capricornutum (P. subcapitata,Green algae)	Freshwater	96-h	4.46 μg/L	Orvos et al. (2002)
P. subcapitata(Green algae)	Freshwater	72-h	0.53 μg/L	Yang et al. (2008)
A. flos-aquae (Blue-green algae)	Freshwater	96-h	1.4 μg/L	Orvos et al. (2002)
A. flos-aquae (Blue-green algae)	Freshwater	96-h	1.6 μg/L	Reiss et al. (2002)
C. ehrenbergii (Green algae)	Freshwater	48-h	620 μg/L	Ciniglia et al. (2005)
D. tertiolecta (Phytoplankton)	Freshwater	96-h	3.5 μg/L	DeLorenzo and Fleming (2008)
S. costatum (Marine diatom)	Seawater	96-h	0.97 μg/L	Orvos et al. (2002)
N. pelliculosa (Golden-brown diatom)	Freshwater	96-h	19.1 μg/L	Orvos et al. (2002)
V. fischeri (Marine bacteria)	Seawater	15-min	0.15 mg/L	Tatarazako et al.(2004)
V. fischeri (Marine bacteria)	Seawater	15-min	0.28 mg/L	Farre et al. (2008)
V. fischeri (Marine bacteria)	Seawater	15-min	0.22 mg/L	Stasinakis et al. (2008)
Activated Sludge microorganisms (SOUR inhibition)	Activated Sludge	3-h	20 mg/L	Orvos et al. (2002)
Activated Sludge microorganisms (glucose uptake rate inhibition)	Activated Sludge	15-min	239 mg/L	Orvos et al. (2002)
Activated Sludge microorganisms (BOD removal rate constant)	Activated Sludge	120-h	1.82 ± 1 mg/L	Neumegen et al. (2004)
Activated Sludge microorganisms (SOUR inhibition)	Activated Sludge	-	38.2 mg/L (θ=5)	Stasinakis et al. (2007)
Activated Sludge microorganisms (SOUR inhibition)	Activated Sludge	-	9.97 mg/L (θ=15)	Stasinakis et al. (2007)
Activated Sludge microorganisms (acute)	Activated Sludge	15-min	2.39×10^5	Samosoe-Petersen et al. (2003)

2.5.1 Effect of TCS on activated sludge systems

The majority (96%) of consumer products containing TCS are eventually rinsed down the drain (Reiss et al., 2002) and discharged with wastewater. Hence its fate after consumed have to known to obtain a detailed information for environment. Although TCS was determined as non-biodegradable in 1990's by OECD 301C and OECD 302C tests it is indicated that it can be extensively mineralized by heterotrophic activated sludge microorganisms by several studies.

Regard to results of obtained experimental studies from literature a minor reduce of (8-10%) COD removal was observed when 0.75 - 2 mg/L of TCS dosed to the systems that contains non-acclimatized biomass. On contrary even a relatively higher concentration such as 2 mg/L of TCS did not affected COD removal of systems which has acclimated biomass. In addition 2 mg/L of TCS affected significantly inhibit ammonia removal and nitrification capacity of non-acclimatized biomass initially (30%). However after an acclimatization period of biomass for 20 days recover of system was observed indicating the rapid acclimatization of autotrophic microorganisms. Moreover the toxicity tests based on the mixed culture (activated sludge) EC_{50} reported between 9.97 and 38.2 mg/L for different sludge ages (θ_c) However this concentrations of TCS can only dissolved in a carrier solvent (methanol), and also this concentration levels are more than solubility of TCS in water.

In addition it is emphasized that some intermediates like methyl- triclosan can occur during the removal process(Federle et al., 2002). During the wastewater treatment process, TCS is transformed by biological methylation into methyltriclosan [MTCS; 5 - chloro - 2 (2,4 dichloropheoxy) anisole; CAS no. 4640-01-1]), methyl-triclosan is a more lipophilic compound (K_{ow} 5.2). This lipophilicity property of methyl-TCS gives resistance against biodegradation process and photolysis so its environmental persistence is greater than its parent compound. Structural formulae of TCS and Methyl-TCS are provided in the figure 2.3.

Although high removal percentages by conventional activated sludge process, still 1-10% of influent TCS and it's metabolites discharged to intake environments. Thus

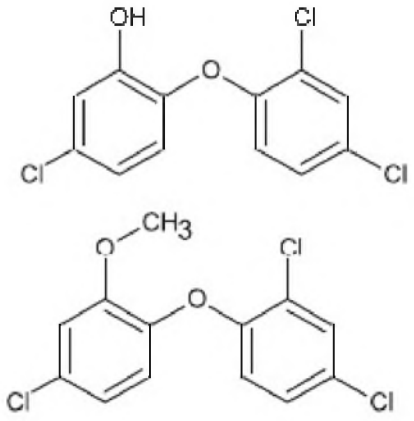

Figure 2.3: Structural formulas of Triclosan (upper diagram) and Methyl-Triclosan (lower diagram) (Bester, 2005)

effluent TCS and its metabolites concentrations have to known to examine its effect on aquatic system.

2.6 Photodegradation

In surface waters light can effect the compounds by photoderived chemical reactions. Light can penetrate through the surface water for several meters from the surface even in the most dark or turbid waters. For the compunds which are found in the surface waters photodegradation (often photodegradation is called photolysis) of compunds are related to the wavelength spectrum and the intensity of the light. For light caused if the energy of the photon is sufficient to break a chemical bond or induce chemical reaction, increased intensity of the light cause the chemical reaction proceed at a faster rate. If the energy need is bigger than the energy of the photon, the chemical bond can not broken by the light regardless of its intensity. Because of the extensive energy that carried by the photons at shorter wavelengths ultraviolet light is mostly effective in degrading many compunds. To cause chemical reactions, the energy possessed by the light must be absorbed by the chemical system. When light is absorbed by an atom it causes to change electrons state from a nonexcited or ground state to excited state. The excited atom or molecule may lose its energy by several ways:

1. The energy may be lost as heat in the process of internal conversion.

2. The electron may lose energy by electromagnetic radiation in the process of florescence.

3. The electron may be changed to its energy level into triplet state. Triplet state may decay radiatively to ground state in the process of phosphorescence.

4. The energy may start a chemical reaction within the molecule in the process of direct photolysis.

5. The energy may be transmitted to another molecule.

For the chemicals which can not absorb energy of protons themselves can be degraded by indirect photodegradation. In the water environment one process of indirect photodegradation is sensitized photodegradation. Sensitized photodegradation may occur when light-absorbing molecules in the water such as plant pigment or humic substances. These molecules are often present in surface waters as dissolved organic matters (DOM) and serve as chromophores. DOM's molecular weight can range from a few hundred to 100.000 Dalton and a large fraction of DOM is consist of humic and fulvic acids. The most comprehensive measurement used to quantify the presence of organic matter in aquatic system is total organic carbon (TOC). Diameter of dissolved fraction of TOC (DOC) is smaller than 0.45 μm thus DOC could be separated by membrane filter which has 0.45 μm pore diameter (Leenheer and Croue, 2003; Hemond and Fechner-Levy, 1991; Dunnivant and Anders, 2006). Most of the variation in humic matter concentration in aquatic ecosystems such as lakes found from 0.5 mg/L to 47 mg/L (TOC) (Kortelainen, 2011).

2.6.1 Photodegradation of TCS

Similarly to other polychlorophenols, TCS can be degraded or converted when exposed to natural sunlight, UV light or heat. However thermal conversion of TCS to the other polycholorodibenzodioxin-*p*-dioxins can be occurred above 300°C. Thus conversion of TCS by heat can not be occurred in surface water or wastewater and heat can be eliminated for degradation of TCS. After identification of 2,8-dichlorodibenzo-*p*-dioxin (2,8 DCDD) in wastewater and surface water samples

and it is hypothesized that 2,8 DCDD could be derived from the photoconversion of TCS (Latch et al., 2003; Chen et al., 2008)

Conversion of TCS to 2,8 DCDD in natural waters investigated by several studies at different pH values. It is observed that TCS can be extensively photodegraded and transformed into 2,8 DCDD ranging from 1-12 % in both pure water and Mississippi River water with UV lamp (Latch et al., 2003). Photodegradation and conversion of TCS into 2,8 DCDD in Mississippi River water can be found in the Figure 2.4:

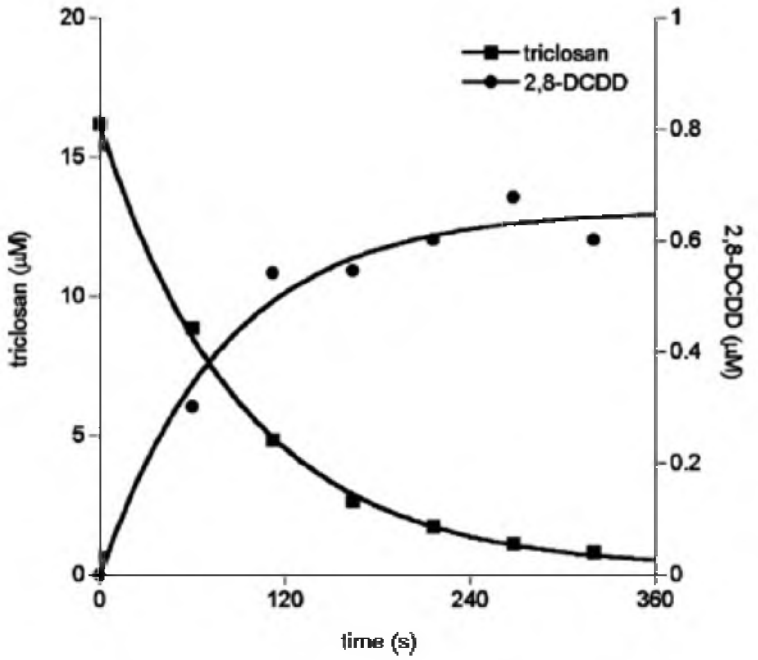

Figure 2.4: Photodegradation of TCS and conversion to 2,8 DCDD in Mississippi River water. (Latch et al., 2003)

Results also tested for confirmation by using TCS containing wastewater and observed that in wastewater samples disappearance of TCS is more rapid compared to deionized water (Mezcua et al., 2004). Changes of TCS and 2,8 DCDD concentration can be seen in the Figure 2.5:

Although earlier studies claimed that no conversion at lower pH values, recent studies demonstrated that photodegradation of TCS and production (please see figure 2.6) of dioxins can be occurred independently of the sample pH. As it can be observed from Figure 2.6 for all different pH's 3, 7.1, and 8.5 degradation of TCS and occurrence of 2,8 DCDD observed but in different rates. This results rejected the hypothesis that dioxins can be only occur when pH is higher. In addition irradiation wavelength plays

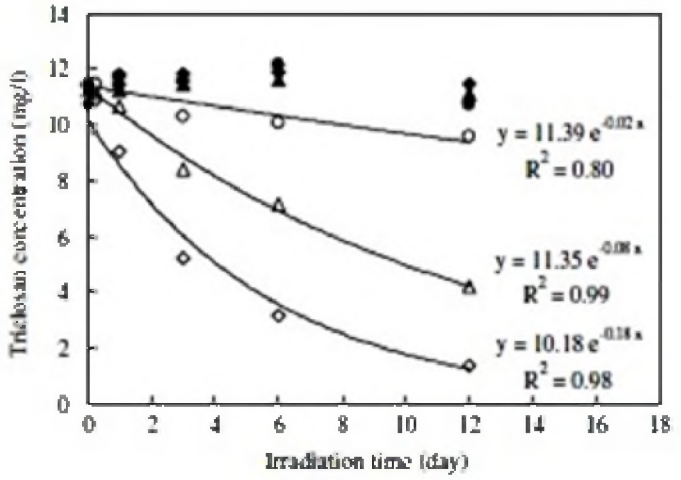

Figure 2.5: Concentration changes of TCS (T, open symbols) and 2,7/2,8-dibenzodichloro-p-dioxin (D, solid symbols) in reagent water and wastewater (Mezcua et al., 2004)

a critical role for photodegradation of TCS (Sanchez-Prado et al., 2006; Sanchez-Prado et al., 2007).

Differently from other results that shown in the Figure 2.8 it is indicated that conversion of TCS to the 2,8 DCDD by florescent light is possible in pure water, fresh water and seawater (Aranami-Readman, 2007).

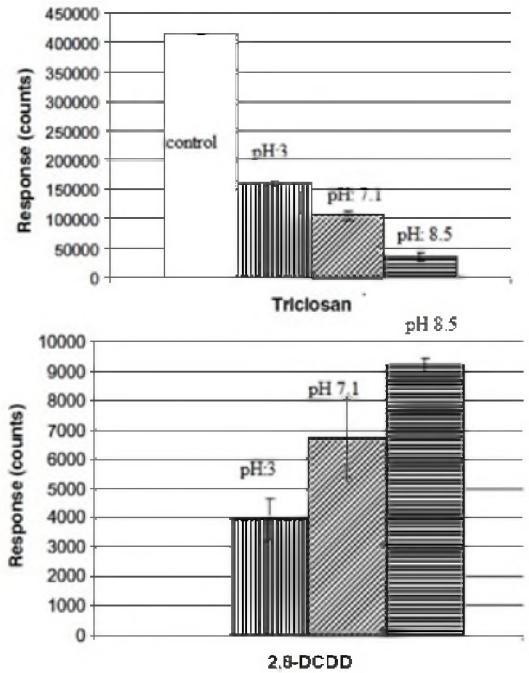

Figure 2.6: Influence of pH on TCS degradation and dioxin generation in wastewater (Sanchez-Prado et al., 2006)

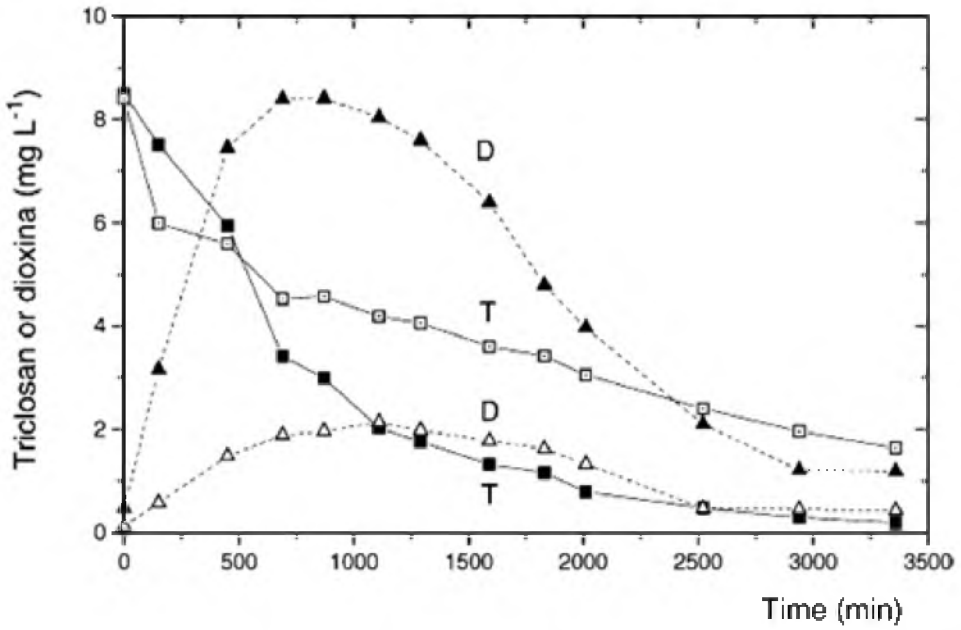

Figure 2.7: Concentration changes of TCS in pure water (circles) freshwater (triangles) and sea water (diamonds) under dark (solid symbols) and light conditions (open symbols) (Aranami and Readman, 2007).

In addition Sanchez-Prado et al (2006) found and identified eight photoproducts of TCS by their mass spectra. Photoproducts of TCS could be generated and reached to its maxima after 20 minutes of irradiation. These photoproducts are listed below:

- 2,8-DCDD
- Dichlorophenol
- Monochlorophenol
- Monochlorohydroxydiphenyl ether
- Dichlorohydroxydiphenyl ether
- Hydroxydichlorodibenzofuran or DCDD

Sanchez-Prado et al (2006) also derived and propose photodegradation pathways (please see figure 2.9) for TCS from the experimental results. Basicly these routes are based on reductive dechlorination to produce dichloro and monochloro-hydroxydiphenyl ethers, reductive photoinduced cyclization to produce DCDD or dichlorohydroxydibenzofuran and photoinduced hydrolysis to produce chlorophenols.

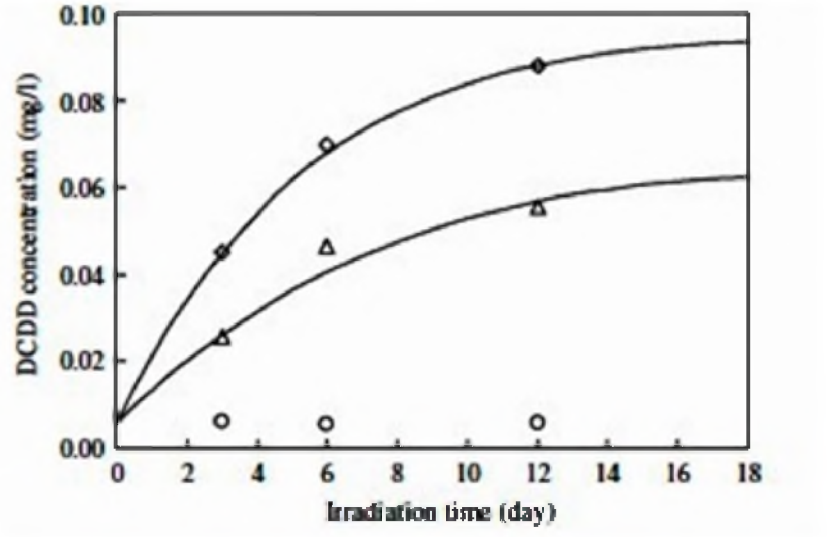

Figure 2.8: Production of 2,8 DCDD in pure water (circles), freshwater (triangles) and sea water (diamonds) under exposure of light (Aranami and Readman, 2007).

It seems to be 2,8-DCDD is a primary photoproduct of TCS and all photoproducts are photodegradable too however their photodegradation rates are different.

Based on the whole reviewed experimental studies after photodegradation of TCS nearly for all different conditions dioxins occurrence was observed. This inference

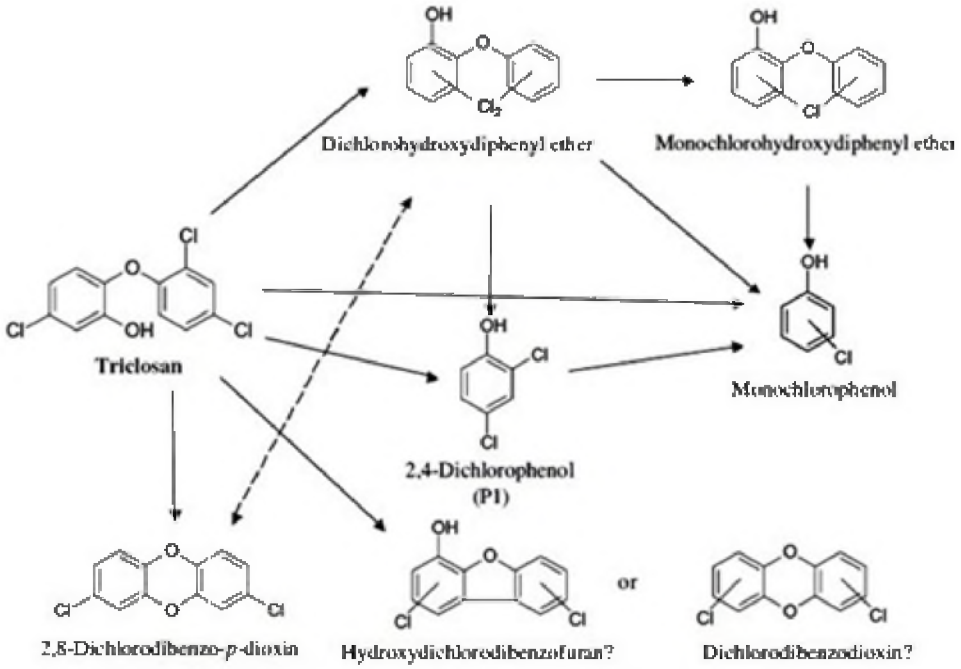

Figure 2.9: Proposed photodegradation pathways of TCS (Sanchez-Prado et al., 2006)

rejected the hypothesis that dioxins could be only occurred at higher pH. Generation of 2,8-DCDD was observed from pH 3 to 9 but higher amount of 2,8-DCDD occurred at higher pH.

Studies indicate that phototransformation of TCS was observed rapidly in natural water than pure water and the organic content of the water effects the conversion (please see figure 2.7). Irradiation wavelength seems to be a critical factor for the conversion of TCS into dioxins. It is found that higher wavelengths cause high yield of dioxins and no significant difference was observed between natural sunlight and solar simulator reactors.

3. MATERIALS AND METHODS

3.1 Reagents and Glassware

All solvents and chemicals were of analytical grade and were purchased either from Merck or Sigma-Aldrich. Distilled water which used for LC/MS analysis was also obtained from Merck.

Standard 250 mL conical flasks were used and the glassware used in the experiments were washed with 1 N HCL and autoclaved at $121^{\circ}C$ for 15 minutes before each experiment set.

3.2 LC-MS/MS Analysis

Electron Cooperation TSQ Quantum Access triple quadruple mass spectrometer coupled with Accela Ultra Performance Liquid Chromatograph (UPLC) in electron spray ionization interface. Samples were directly injected into the mass spectrometer using the sample loop and were analyzed in full scan mode.

3.3 Toxicity Tests

3.3.1 Algal growth inhibition experiments with marine algae

P. tricornutum was selected as an algae species to examine toxicity of TCS to the marine algae species. The microalgae culture was acquired from the Ecotoxicology Laboratory which presents in Faculty of Naval Architecture and Ocean Engineering of Istanbul Technical University. Cultures was grown in flasks which have volumes of 250 mL for each. Flasks were maintained under continuous cool-white florescent light at 20 $\pm$ 1°C. For the toxicity tests 0.4 μg/L and 4 μg/L samples prepared with a control and duplicated to obtain any toxic effect evidence of TCS. Seawater sample taken from Istanbul Bosphorus and filtered through an activated carbon filter to remove possible organic contaminants. After that filtered seawater was filtered again by a GFC

Table 3.1: Content of stock solutions for growth media of *Phaeodactylum tricornutum* (Guillard and Ryther, 1962)

Stock Solution	Nutrient	Mass Concentrations
Stock 1: $NaNO_3$	$NaNO_3$	75 g
Stock 2: Na H_2PO_4	$NaH_2PO_4 \cdot 2H_2O$	5.66 g
Stock 3: Na_2SiO_3	$Na_2SiO_3 \cdot 5H_2O$	12.9
Stock 4: Trace Metals	$CuSO_4 \cdot 5H_2O$	0.005 g
	$ZnSO_4 \cdot 7H_2O$	0.011 g
	$CoCl_2 \cdot 6H_2O$	0.005 g
	$MnCl_2 \cdot 4H_2O$	0.009 g
Stock 5	$FeCl_3 \cdot 6H_2O$	0.909 g

(Glass-fiber) filter paper which have 0.2 μm pore diameter. Double filtered seawater was autoclaved (Hirayama HV 50 L) for 10 minutes at 110°C for sterilization. Growth media was prepared in the double filtered and autoclaved seawater and enriched with Guillard's f/2 nutrient medium (Guillard and Ryther, 1962).

To prepare growth media in 100mL of seawater, 0.12 mL of stock solutions were taken from each stock. 0.6 mL algae was added to the 100 mL growth media to adjust final algal concentration in test volume to $1x10^4$ cell/mL. Experiment period was determined as 7 days. The chlorophyll florescence of the algae was measured by a florescence spectrophotometer (Perkin Emler LS 55) at excitation and emission wavelengths of 430 nm and 663 nm respectively at the beginning and the end of period. Inhibition rates were calculated by using the same formula that explained before in the toxicity experiments with *P. Subcapitata.*

3.3.2 Algal growth inhibition experiments with freshwater algae

Determination of toxicity of TCS to the freshwater species was examined by algal growth inhibition experiments. *P. subcapitata* was selected as test species and experiments were designed regard to EN ISO 8692:2004 with minor modifications and algal culture (Sammlung von Algenkulturen) was obtained from University of Göttingen. Four different nutrient solutions were prepared according to the table below, in deionized water (electric conductivity: 0.055 μS/ cm) and autoclaved at 121°C for 15 minutes except the 4^{th} stock solution.

Table 3.2: Mass concentrations of nutrients in the test solution (EN ISO 8692:2004)

Stock Solution	Nutrient	Mass concentration in stock solution	Final Mass concentration in test solution
Stock Solution 1: Macro Nutrients	NH_4Cl	1.5 g/L	15 mg/L
	$MgCl_2$	1.2 g/L	12 mg/L
	$CaCl_2$	1.8 g/L	18 mg/L
	$MgSO_4 \cdot 7H_2O$	1.5 g/L	15 mg/L
	KH_2PO_4	0.16 g/L	1.6 mg/L
Stock Solution 2: Fe-EDTA	$FeCl_3 \cdot 6H_2O$	64 mg/L	64 μg/L
	$Na_2EDTA \cdot 2H_2O$	100 mg/L	100 μg/L
Stock Solution 3: Trace Elements	H_3BO_3	185 mg/L	185 μg/L
	$MnCl_2 \cdot 4H_2O$	415 mg/L	415 μg/L
	$ZnCl_2$	3 mg/L	3 μg/L
	$CoCl_2 \cdot 6H_2O$	1.5 mg/L	1.5 μg/L
	$CuCl_2 \cdot 2H_2O$	0.01 mg/L	0.01 μg/L
	$Na_2MoO_4 \cdot 2H_2O$	7 mg/L	7 μg/L
Stock sloution 4: $NaHCO_3$	$NaHCO_3$	50 g/L	50 mg/L

The 4^{th} solution was sterilized by the membrane filter which has 0.2 μm pore diameter. Growth media was prepared in 1 L of deionized and autoclaved water by taking 10 mL from Stock 1 solution and 1 mL from Stock 2, Stock 3 and Stock 4 solutions. Algal toxicity tests were carried out in 250 mL conical flasks containing 100 mL of algal growth media. Each flask was covered with aluminium foil to eliminate contamination risk and the foil was perforated to allow gas exchange. Control and test flasks are inoculated with 5 mL algae which were in exponentially growth phase. Cultures were maintained in a cabinet under continuous illumination (white florescent light) at 22 $\pm$ 4°C for incubation. To obtain a EC_{50} value a wider range of TCS concentrations were tested for 14 different experiments. Each flask stirred once a day due to lack of an orbital shaker under florescent light. For experiments No: 1 to No: 6 algal growth yield was measured by optical density of the 10 cm cell suspension at wavelength 630 nm by a spectrophotometer (Pharmacia LKB, Novospec II, England) For the experiments No: 7 to No:10 algal growth yield was measured by a hemocytometer. The concentrations used in the tests were nominal concentrations and did not measured by any instruments.

Calculation of inhibition percentage could be done by the following formulae. Firstly average specific growth rate have to calculated:

$$\mu = \frac{ln(x_L) - ln(x_0)}{t_L - t_0} \tag{3.1}$$

Table 3.3: Tested concentrations of TCS to observe algal growth inhibition

Experiment No	Tested Concentrations (μg/L)
1	0.1 - 0.2 - 0.4 - 0.8
2	0,8 - 2 - 4 - 8
3	0,4 - 4
4	0,8 - 2 - 4 - 8
5	0,4 - 4
6	0.1 - 0.2 - 0.4 -0.6 - 0.8
7	32 - 64 - 125 - 250 - 500 - 1000 (3 h UV exposed)
	32 - 64 - 125 - 250 - 500 - 1000
8	32 - 64 - 125 - 250 - 500 - 1000 (3 h UV exposed)
	32 - 64 - 125 - 250 - 500 - 1000
9	32 - 64 - 125 - 250 - 500 - 1000 (3 h UV exposed)
	32 - 64 - 125 - 250 - 500 - 1000
10	1 - 5 - 10 - 20 - 50 - 100
11	50 - 20 - 10 - 5 - 1
12	0.5 - 1 - 2.5 - 5 - 7.5 - 10
13	0.5 - 1 - 2.5 - 5 - 7.5 - 10
14	2.5 - 5 - 7.5 - 10 - 15 - 20 (3 h UV exposed)
15	2.5 - 5 - 7.5 - 10 - 15 - 20 (3 h UV exposed)

Where;

t_0: time of test start

t_L: time of the last measurement within the exponential growth period in the control cultures

x_0: nominal initial cell density

x_L: measured cell density at time t_L

Mean value of μ calculated for each test and control batch replicate. From μ values percent inhibition for the each test and control batch replicate could be calculated by :

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100 \qquad (3.2)$$

Where;

$I_{\mu i}$ is the percentage inhibition (growth rate) for test concentration,

μ_i is the mean growth rate for test concentration,

μ_c is the mean growth rate for control.

Inhibition data obtained from the all flasks tabulated and plotted for all test concentrations regard to the normalised inhibition against the test concentration on a logarithmic scale. Experimental data points fitted to a suited non-linear model to the experimental data points by regression analysis in order to determine EC_{50} values.

3.4 *V. fischeri* Toxicity Experiment

Principle of the assay can be determined as the inhibition of the luminescence marine bacteria by different dilutions of the target toxic samples. To perform bioluminescence bioassay dry freezed *V. fischeri* toxicity kits were purchased from Sigma-Aldrich®.

Reagents and instruments:

- Luminometer (ABOATOX 1253)
- Luminometer cuvettes
- +15°C dry block incubator
- Solid 1243-551 NaCl
- 1 ml and 5 ml pipettes and pipette tips
- 1257-130 BioTox™ Software

Assay procedure was conducted according to instructions of BO1243-500 BioTox™. To obtain 20% NaCl solution 1243-559 BioTox™ NaCl solid tablet was dissolved to 45 mL of double distilled water (electric conductivity: 0.055 μS/cm). 4 mg/L stock TCS solution was prepared and pH control was conducted. The final salinity of the sample adjusted to 2% by adding NaCl solution. The 1243-551 BioTox™ Reagent Diluent was melted and cooled to +4°C in a cooler then *V. fischeri* Reagent reconstituted by adding the contents of the reagent. The reconstituted reagent was equilibrated at +4°C in cooler for 1 hour. Then the reagent stabilised at +15°C in a incubator for 45 minutes before pipetting into the cuvettes. 500 μL of reagent pipetted into the cuvettes and stabilised 15 minutes at +15°C in incubator. Sufficient volume

of 1243-559 BioTox™ NaCl solution was diluted 1:10 with double distilled water to obtain 2% NaCl. Sample dilution series were prepared and their salinity were adjusted to 2% by using NaCl solution. Sample dilution ratios were 1:1, 2:3, 1:2, 1:3, 1:4, 1:6 and 1:8 respectively thus to measure EC_{50} value of TCS to *V. fischeri* stock solution was diluted 1:2 to adjust its TCS concentration to 2 mg/L. 10 ml sample was taken and to adjust the salinity 1.1 mL of 20% NaCl solution was added. Various concentrations of TCS were diluted as 1.8, 1.2, 0.9, 0.6, 0.45, 0.3, 0.225 and 0 mg/L respectively. All dilutions and control cuvettes were prepared duplicate and maintained at +15°C The luminescence intensity (I_0) were measured from the first cuvette containing bacterial suspension. After measuring the I_0, 500 μL of samples were added to the each cuvette immediately. 15 minutes of incubation period selected as contact time. After contact time accomplished luminescence intensities (I_t) of each cuvettes measured again by using the luminometer. Measurement repeated for all samples using the same time interval as during the first measurement. BioTox™ Software was used for adjusting the time intervals and calculation of the inhibition rates. BioTox™ Software calculates the inhibition percentages by using the formulae below:

$$KF = \frac{IC_{15}}{IC_0} \tag{3.3}$$

$$Inh\,(\%) = 100 - 100 \times \left[\frac{IT_{15}}{KF \times IT_0}\right] \tag{3.4}$$

Where;

KF = Correction factor

IC_{15} = Luminescence intensity of control after contact time (15 min.) in Relative Luminescence Unit (RLU)

IC_0 = Initial luminescence intensity of control sample in RLU

IT_{15} = Luminescence intensity of test sample after contact time (15 min.) in RLU

IT_0 = Initial luminescence intensity of the test sample in RLU

Results noted as text files and EC_{50} values were calculated by 3 parameter sigmoidal non-linear regression analysis and dose-response curves are plotted within the 95% confidence interval by using SigmaPlot 12.0 software.

3.5 *V. fischeri* Toxicity Experiment After Photolysis of Triclosan

Experiment procedure was conducted according to instructions of BO1243-500 BioTox™ as the same as explained previously. To examine toxicity of photoproducts of TCS to *V. fischeri* 4 mg/L stock TCS solution was exposed to UV light in a quartz glass which allows UV transition for 3 hours. Stock TCS solution was not diluted to obtain statistically more robust measurement data. Test TCS concentrations were 0.45, 0.6, 0.9, 1.2, 1.8, 2.4 and 3.6 respectively. In order to simulate realistic seawater conditions 12 mg/L humic acid solution was prepared in double distilled water to obtain 5 mg/L total organic carbon containing solution. Prepared solution was filtered through the filter which has 0.45 μm pore diameter and TCS was added to obtain 4 mg/L of TCS concentration. Humic acid solution and humic acid-TCS solution was exposed to the UV light for 3 hours separately. *V. fischeri* suspension was exposed to humic acid and UV exposed humic acid solution as control. Experiment was conducted regard to explained procedure before.

3.6 Activated Sludge Toxicity Experiments

Respirometric tests were conducted to examine toxicity of TCS to mixed culture biomass of activated sludge. Activated sludge sample was taken from a reactor fed with peptone that have 400 mg/L COD. 1600 mL activated sludge was taken from the reactor has a 8 L volume and settled to 400 mL. 10 mL macronutrient and 10 mL micronutrient solutions were added to the settled sludge. For the control total test volume was fulfilled to 2000 mL with tap water and for the test system volume was fulfilled with the predetermined concentrations of TCS solution. Final TCS concentrations in the test equipment were adjusted to 0.8 mg/L , 1.6 mg/L and 6 mg/L respectively. Initial MLVSS concentrations were 850 and 1150 mg COD/L. At the beginning of the experiment internal respiration rates have been observed for 10 minutes then 27 mL of peptone (COD = 400 mg/L) and meat extract were added as substrate to the activated sludge in the respirometry equipment (Applitek RA-1000).

To eliminate oxygen consumption of autotrophic growth nitrification inhibitor was added to the reactor (Formula 2533^{TM} -Hach Company). Respirometric instrument was consist of a 5-L Plexiglas reactor and a second reactor with a 0.75 L volume where oxygen utilization rates (OUR) are measured by a continuous respirometer (Manotherm RA-1000) with PC connection. Activated sludge is circulated with a sample pump during the whole experiment and OUR was measured continuously with a period of 1 minutes. Flow rate of the air was adjusted to approximately 8-9 mg/L of O_2 at the beginning of the experiment. Measurements were conducted for 15 hours till the whole substrate was consumed. Respirometric results were modeled Activated Sludge Model (ASM 1) with Aquasim Software.

4. RESULTS

4.1 *V. fischeri* Toxicity Experiments

In order to determine toxicity of TCS, luminescent marine bacteria *V. fischeri* was selected and Microtox® tests were performed in. Thus all experimental results are duplicates. TCS solutions were prepared initially as 0, 0.09, 0.12, 0.18, 0.24, 0.36, 0.48 and 0.72 mg/L and after the initial test a different range was used as 0, 0.225, 0.3, 0.45, 0.6, 0.9, 1.2, and 1.8 mg/L respectively to obtain a more robust EC_{50} value and experiments performed in duplicate. After 15 min contact time detoriation of light emission values were plotted on a dose response curve. In that way acute toxicity of TCS to *V. fischeri* was evaluated. Dose response curves obtained for different concentration ranges are shown in Figure 4.1.

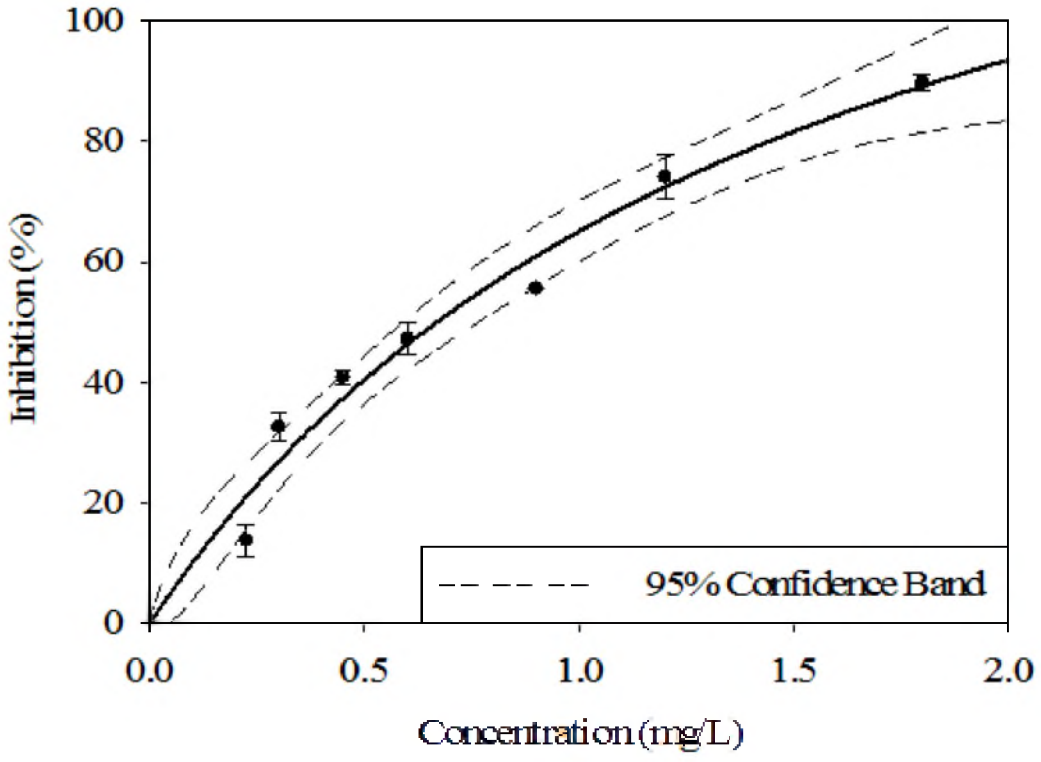

Figure 4.1: Inhibitory effect of TCS on light emission of *V. fischeri*

EC_{50} value was calculated as 0.67 mg/L by 4-parameter sigmoidal non-linear regression analysis.

4.2 *V. fischeri* Phototoxicity Experiments

Although TCS is a photodegradable compound, studies about the toxicity of photoproducts of TCS are scarce. The effect of photolysis on the toxicity of TCS was evaluated by performing phototoxicity experiments by using an UV cabinet under 4500 lux of light intensity for 3 hours. Based on literature studies on the toxicity of photoproducts of, a decrease in the toxic effect was assumed, thus higher concentration of TCS solutions were used as 0, 0,45, 0.60, 0.90, 1.20, 1.80, 2.40 and 3.60 mg/L respectively. Dose response curves obtained from the Microtox ® experiment are shown in the Figrue 4.2.

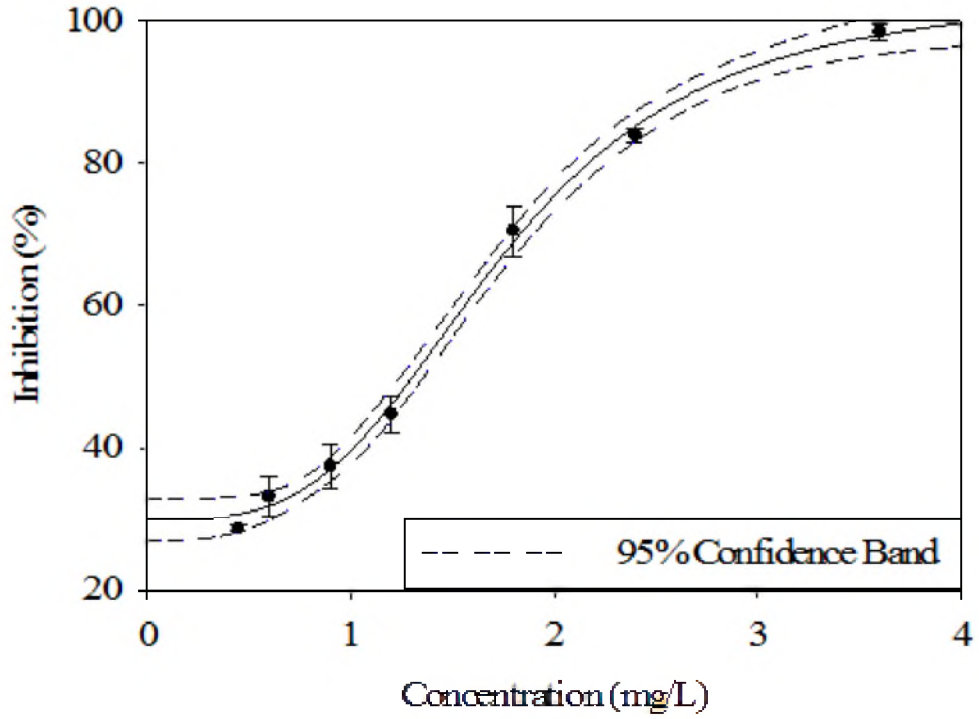

Figure 4.2: Dose-response curves of TCS after irradiation of TCS by UV-A for 3 hours

Different from the prior experiment, median EC_{50} value was calculated as 1.29 mg/L by 4 parameter sigmoidal non-linear regression analysis.

Increase in the EC_{50} values after photolysis agree with the qualitative measurements obtained with the LC/MS. After exposing TCS to UV for 3 hours, the initial signal intensity of m/z 287 corresponding to TCS decreased approximately 40%,

Table 4.1: Effect of humic acid solution and UV exposed Humic Acid solution on light emission rate of *V. fischeri*. Reducing of the light emission rates can be subjected to the dilution of suspension including *V. fischeri*.

Humic Acid Control	I_0 (RLU)	I_1 (RLU)	Reducing of Light Emission
5 mg/L TOC Equivalent Solution	254400	160700	37%
5 mg/L TOC Equivalent Solution (3h UV exposed)	248300	153300	38%
NaCl Control	242100	154900	37%

demonstrating the degradation of TCS by UV irradiation. More on the LC-MS/MS results along with the figures will be provided after the toxicity test results. In addition TCS exists in surface water usually together with humic substances as dissolved organic matters. These organic substances may lead to indirect photodegradation which results in another toxic compounds or complete photodegradation of TCS. To simulate surface water conditions humic acid was selected as organic matter and solution prepared according to typical concentrations of surface waters. As selected DOM humic acid-TCS solution and only humic acid solution equivalent to 5 mg/L dissolved organic matter were irradiated for 3 hours Humic acid solution was used as control to determine humic acid's effect on light emission rate of *V. fischeri*. Control test results are shown in the Table 4.1.

As control test results demonstrate both 5 mg/L TOC equivalent humic acid solution and its 3 hour photolyzed form did not detoriorate the light emission. Reduction of light emission is not significantly different from the control test result performed with NaCl that is within the normal Microtox ® test procedure.

Experiment results are slighlty different from the results of phototoxicity tests performed with TCS solution which does not contains humic acid solution. Dose response curves of the experiment performed with the 5 mg/L TOC equivalent solution and as 0, 0.45, 0.60, 0.90, 1.20, 1.80, 2.40 and 3.60 mg/L TCS solution are shown in the Figure 4.3.

EC_{50} value was calculated as 1.37 mg/L by 4-parameter sigmoidal non-linear regression analysis. Effect of UV and presence of humic substances on EC_{25} and EC_{50} values are provided in the figure 4.4.

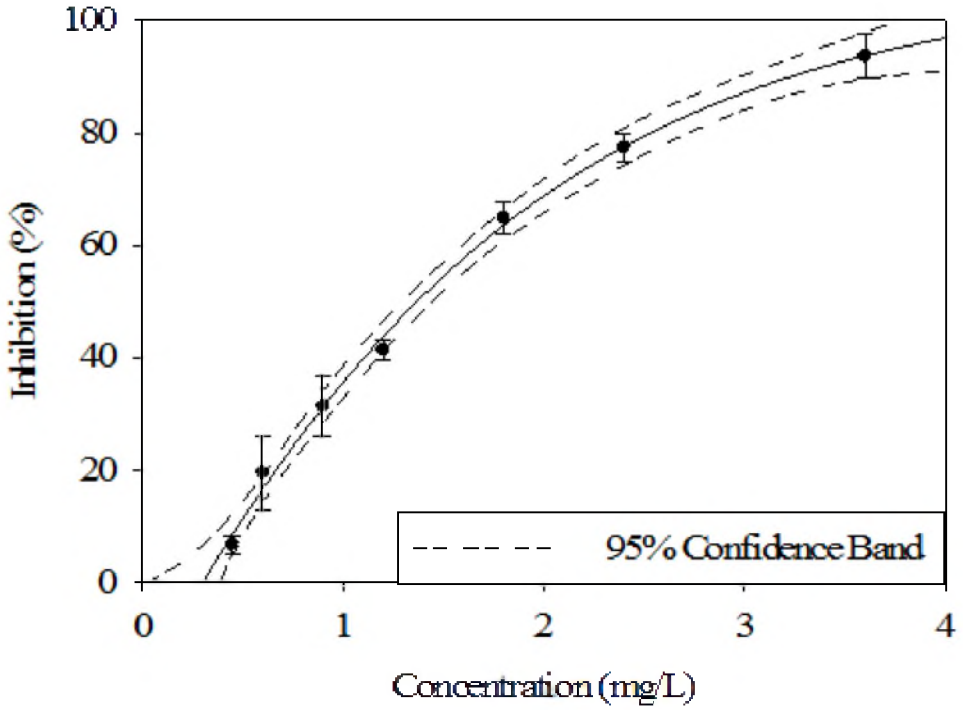

Figure 4.3: Dose-response curves of 0, 0.45, 0.60, 0.90, 1.20, 1.80, 2.40 and 3.60 mg/L + 5 mg/L TOC equivalent humic acid solution after irradiation of TCS by UV-A for 3 hours.

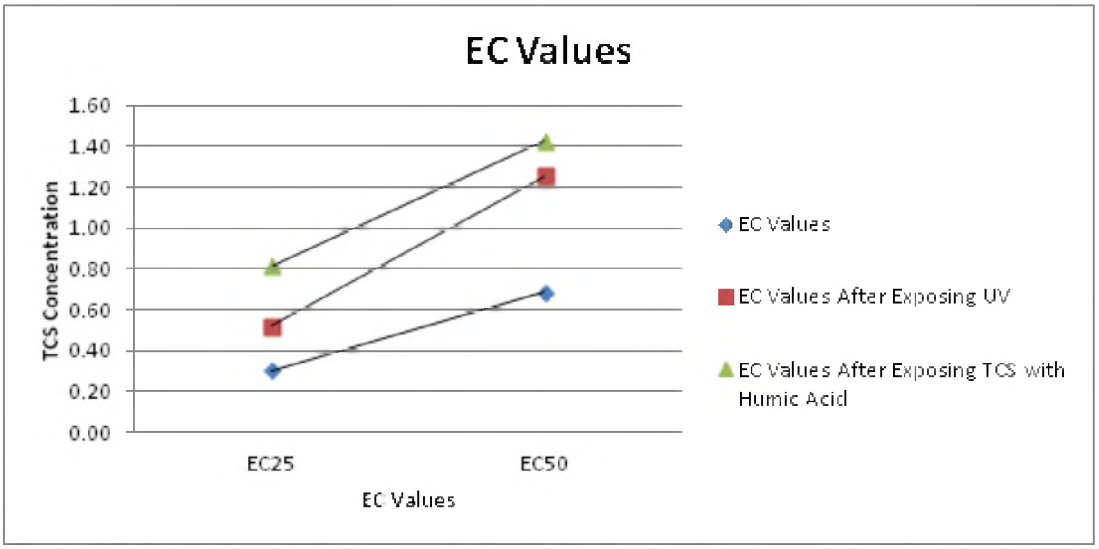

Figure 4.4: EC_{25} and EC_{50} values for *V. fischeri* and effect of 3 hour UV exposure and 5 mg/L TOC equivalent humic acid on the obtained values

This results indicate that the presence of humic substances during photodegradation contributes to advanced degradation of TCS (64%). However EC_{50} value of *V. fischeri* increased 51% indicating the probable toxic effect of the photoproducts of TCS. In environmental waters, TCS will exist together with naturally occurring organic matter

or humic substances and therefore, they will be subjected to indirect photolysis leading to reduced toxic effects to *V. fischeri*.

4.3 *P. tricornutum* Toxicity Experiments

Samples containing 0.4 μg/L and 4 μg/L TCS were prepared and toxicity tests in duplicates along with a control were conducted to evaluate the possible toxic effect of TCS to *P. tricornutum*. Experiment period was 7 days for both concentrations. The chlorophyll florescence of the algae was measured by a florescence spectrophotometer at excitation and emission wavelengths of 430 nm and 663 nm, respectively, at the beginning and at the end of the experiment period. At these concentrations TCS did not adversely effect the growth of *P. tricornutum*.

4.4 *P. subcapitata* Toxicity Experiments

Determination of toxicity of TCS to the freshwater species was examined by algal growth inhibition experiments using *P. subcapitata*. Several initial experiments were conducted to observe an inhibition in the growth of *P. subcapitata* since there were conflicting studies on the concentrations that show a growth inhibition (Orvos et al., 2002; Tatarazako et al.,2004; Orvos et al., 2002; Yang et al., 2008). 0, 0.5, 1, 2.5, 5, 7.5, 10 μg/l TCS solutions were used to obtain an EC_{50} value. Experiments were carried out for the 72 hour-growth period. At the end of the test period, algal community was counted by hemocytometer under microscope. The dose-response curve obtained in the experiments is provided in Figure 4.5.

From the results of regression analysis, the median EC_{50} is obtained as 7.74 μg/L for *P. subcapitata*.

4.5 *P. subcapitata* Phototoxicity Experiments

Similar to the experiments on *V. fischeri*, the effect of UV light on the toxicity of TCS to *P. Subcapitata* was investigated based on the obtained results on *V. fischeri* which showed reduced toxicity due the photolysis. Higher concentration of TCS solutions were used as 0, 2.5, 5, 7.5, 10, 15, 20 μg/L. These concentrations were obtained by

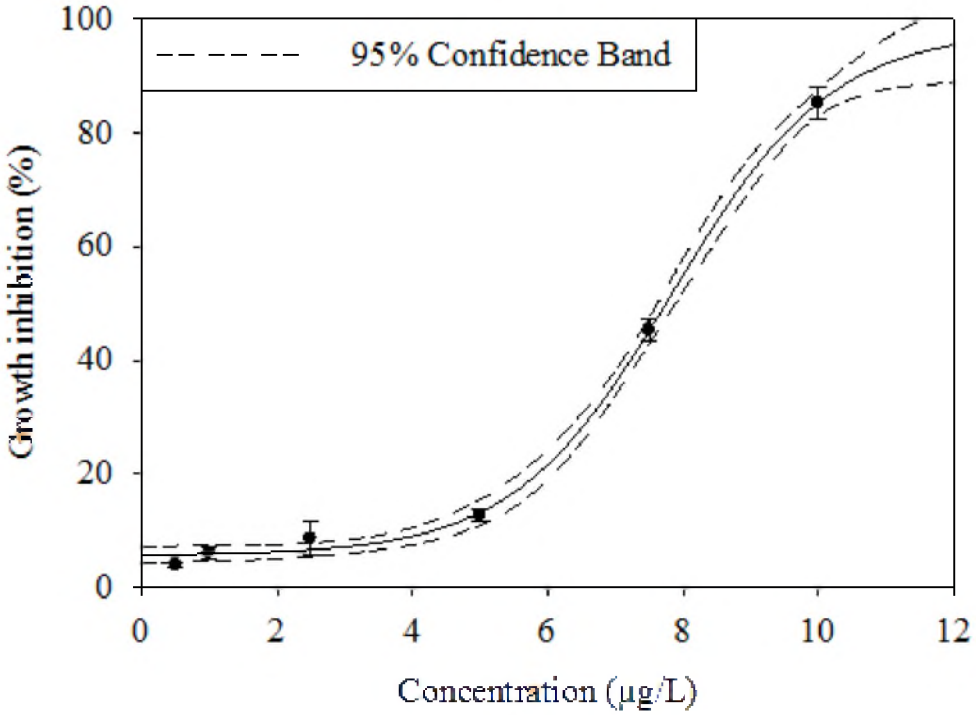

Figure 4.5: Dose response curves obtained from the 72 hour algal growth inhibition test by using 0, 0.5, 1, 2.5, 5, 7.5, 10 μg/l TCS solutions

diluting a stock solution (400 μg/L) which was exposed to UV-A light for 3 hours. The dose-response curve obtained in the experiments is provided in Figure 4.6.

The median EC_{50} for UV-exposed TCS was obtained as 8.70 μg/L from the results of 5 parameter sigmoidal non-linear regression analysis for *P. subcapitata*. The effect of photolysis on the toxicity can be evaluated by observing the change in the EC_{50} values. The increase in EC_{50} value indicates a decrease in toxicity upon photolysis. Moreover, EC_{10} and EC_{25} and EC_{80} values can be used for a more thorough analysis. For algae, the toxicity decreases slightly due to photolysis based on EC50 values but this decrease is statistically significant at a level of 0.95. The changing EC_{10}, EC_{25}, EC_{50}, and EC_{80} values are provided in Figure 4.7. The EC_{10} and EC_{25} values indicate that the no observed/lowest observed effect levels of TCS and photolyzed TCS are rather different and the photolysis products seems to affect a higher percentage of the population at concentrations lower than 8 μg/L. This result is significant since the concentration of TCS in the surface waters is at this level and surface waters are subject to photolysis. As a summary, all EC values are provided in the table 4.2.

Table 4.2: Summary of EC_{50} values of *V. fischeri* and *P. subcapitata*

Species	Method	EC_{50} (95% Conf. Int.)
V. fischeri	15 min. contact	0.67 mg/L
V. fischeri	15 min. contact + 3h UV	1.29 mg/L
V. fischeri	15 min. contact + 3h UV + Humic Acid	1.37 mg/L
P. subcapitata	72h algal growth inhibition	7.74 μg/L
P. subcapitata	72h algal growth inhibition + 3h UV	8.7 μg/L

4.6 LC-MS/MS Results

Literature suggests that 2,4-dichlorophenol and 2,8-DCDD are the main photoproducts of TCS (Latch, 2005). The effect of UV light has been studied using LC-MS/MS in full scan mode. The signal intensity of m/z:287 indicating TCS ($M\text{-}H^+$), decreases approximately by 40% when 4 mg/L of TCS is exposed to 3 h UV treatment indicating that UV degrades TCS (Figure 4.8 and 4.9).

A similar decrease (approximately 30%) in TCS concentration upon 3h photolysis has been reported in the literature (Martinez-zapata, 2013). The decrease in TCS

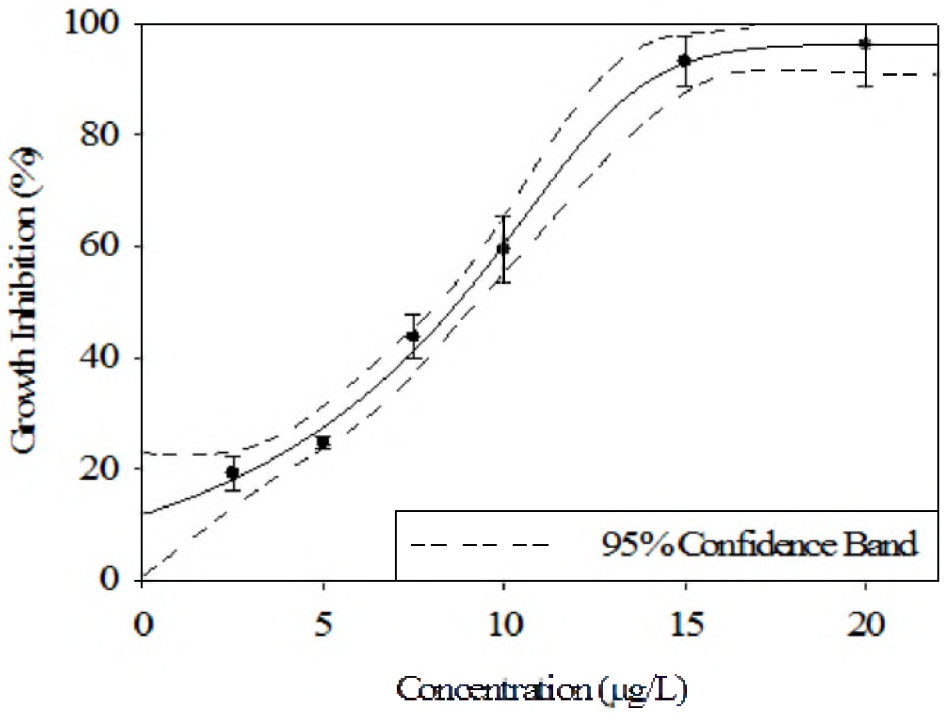

Figure 4.6: Dose response curve obtained from the 72 hour algal growth inhibition test by using 0, 2.5, 5, 7.5, 10, 15, 20 μg/L TCS solutions after exposed to UV-A for 3 hours. (Dashed lines show the 95% confidence interval.)

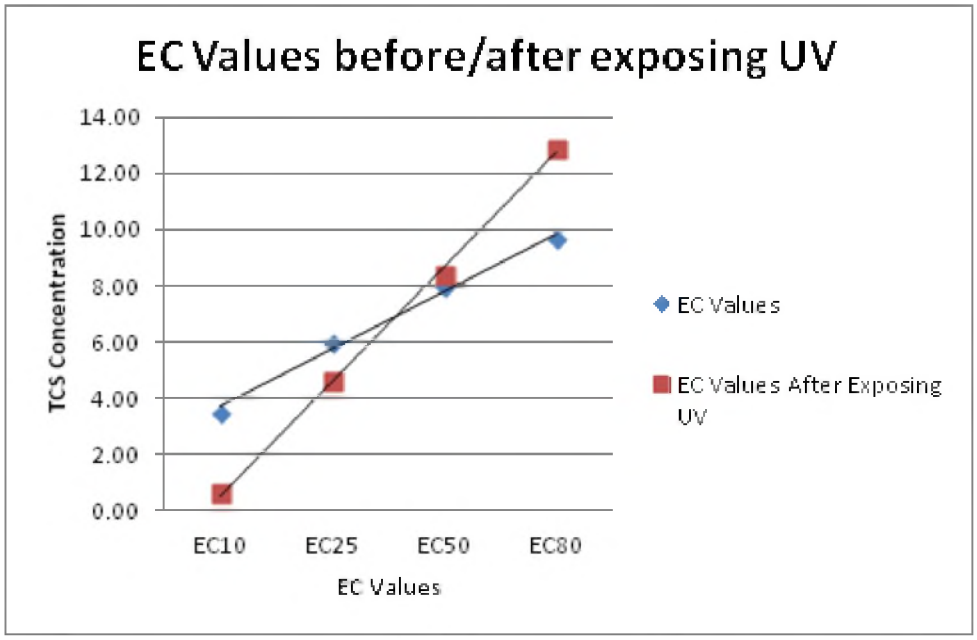

Figure 4.7: EC_{10}, EC_{25}, EC_{50}, and EC_{80} values for *P. subcapitata*

concentration results is in agreement with the increase in EC_{50} values upon UV treatment. The full scan of the UV-treated sample containing only 4 mg/L TCS has several ions with m/z of 205.13 and 234.95 as well as 302 suggesting that there are several photodegradation products of TCS (Figure 4.9). Among these possible photodegradation products, m/z 235 can be formed upon chlorine loss from TCS (Ferrer, 2004). The ion with m/z of 303 could be the results of the coupling of two chlorine to TCS which is the results of photoionization (Wong et al., 2007).

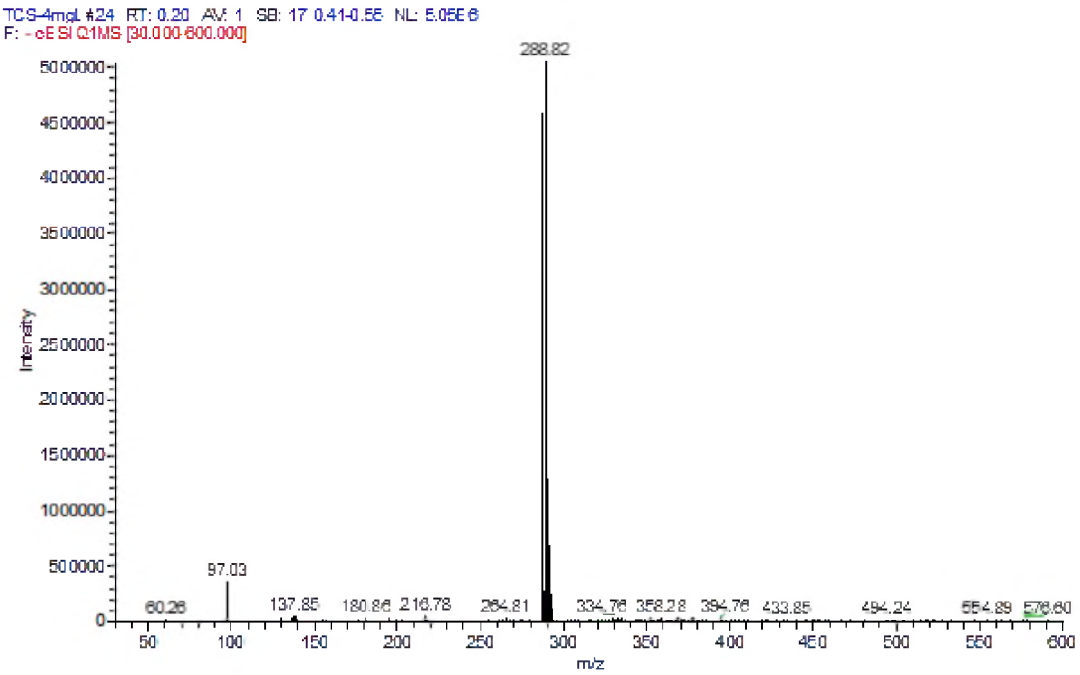

Figure 4.8: Mass spectrum of 4 mg/L TCS

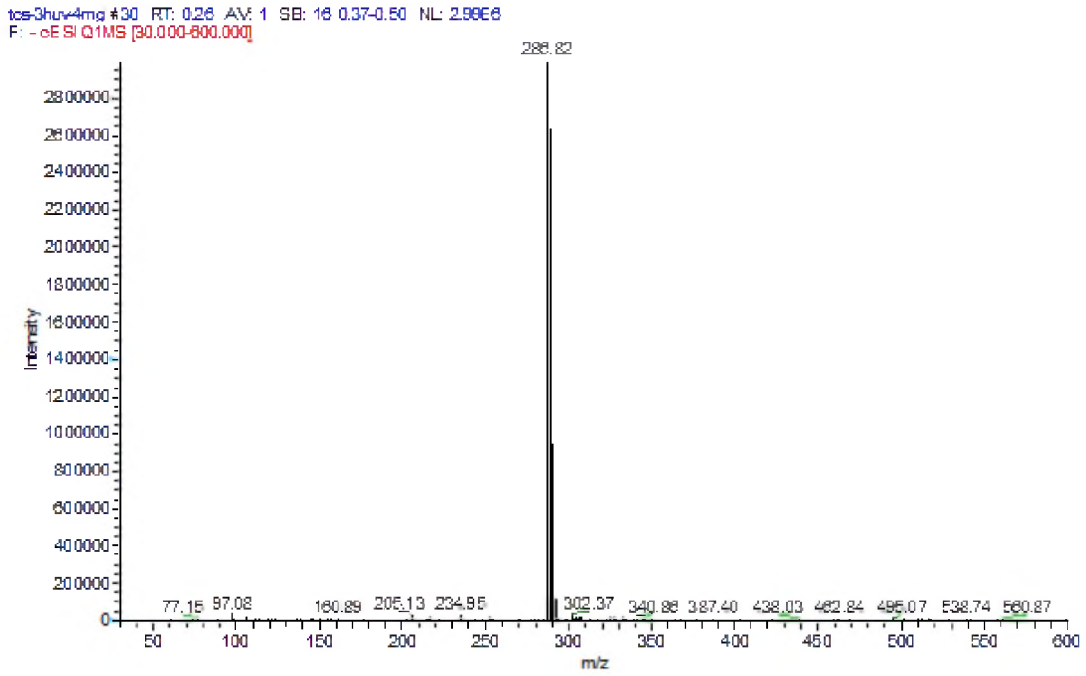

Figure 4.9: Mass spectrum of TCS after 3 hour UV exposure

Similarly, the full scan of the UV-treated sample containing 4mg/ L TCS and HS (Figure 4.10) has several ions with m/z of 144.86, 204.96 and 255.03 suggesting that there are several photodegradation products of TCS during indirect photolysis. The presence of HS and the ions originating from it make it harder to detect and/or distinguish the photoproducts since different molecules might have to the same m/z.

Although the literature suggest the presence of an ion with m/z of 253.3 or 255 as a possible photoproduct of TCS, the observed 255.03 in our samples is probable not a photodegradation product of TCS but is actually originating from the HS since both the unphotolyzed (Figure 4.11) and photolyzed HS solutions (Figure 4.12) contain this ion whereas the direct photolyzed solution does not contain this ion.

2,4 DCP (m/z: 189) results from the breaking up of the TCS molecule in half and then the subtraction of OH. However, this ion is not observed in our samples neither in direct nor in indirect photolysis. Although the m/z of 145 exist in the full scan of the sample containing only HS, the HS subjected to UV does not include this ion, suggesting that this m/z belongs to an ion that originates from HS. Therefore, we can say that in the UV- treated sample with TCS and HS, the m/z of 145 is indeed a photodegradation product of TCS. Similarly, an ion with m/z 146 has been observed during chemical ionization of photolyzed TCS (Sanchez-Prado et al., 2006). The difference between 145 and 146 arises from the different ionization techniques used

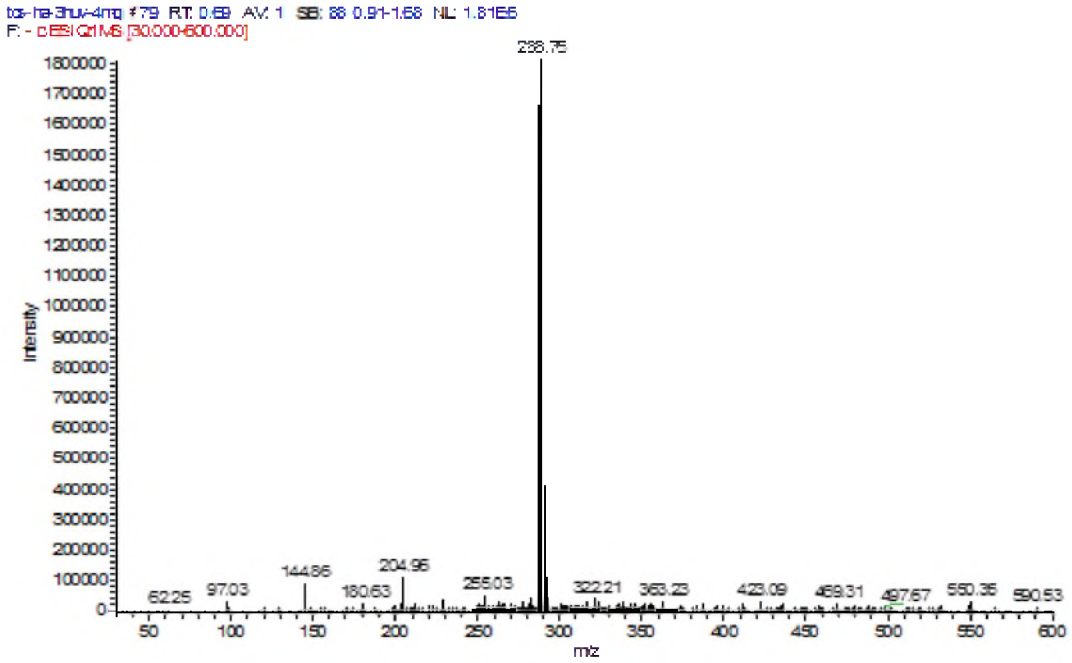

Figure 4.10: Mass spectrum of UV-treated sample containing 4mg/L TCS and HS

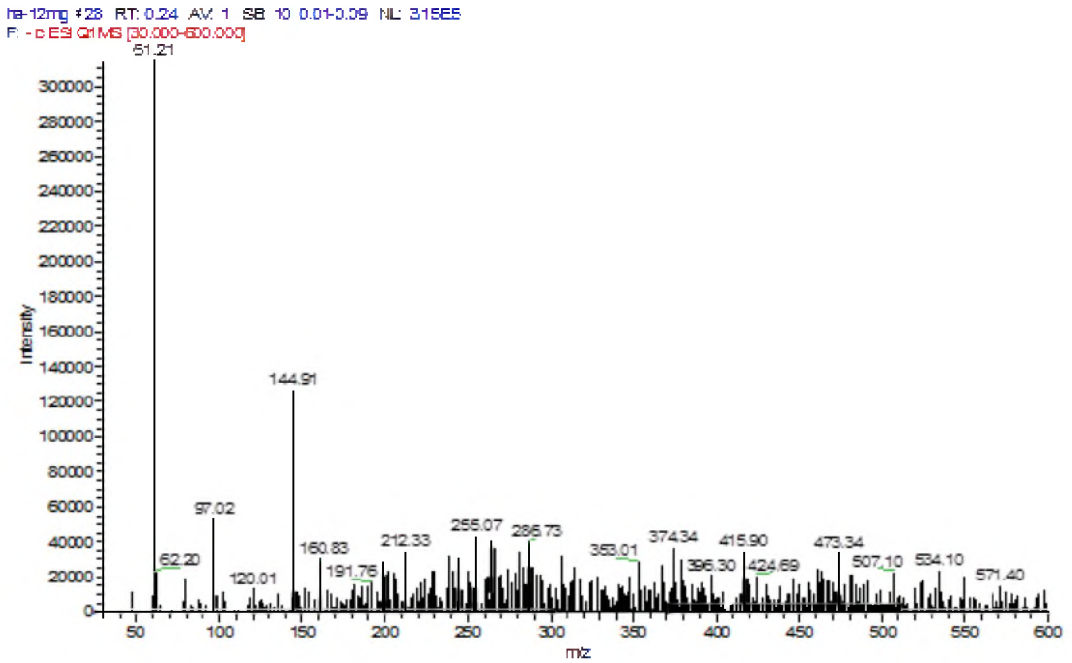

Figure 4.11: Mass spectrum of HS

which result in either no loss of H^+ or loss of H^+. Although normally a m/z is a good indicator for a compound and should not change, there are studies that suggest the m/z for TCS and its photodegradation by-products have a range of m/z values rather than one m/z (Sanchez-Prado et al., 2006). The use of high resolution-MS can provide more accurate information on the possibility of different fragmentations/ionization of TCS.

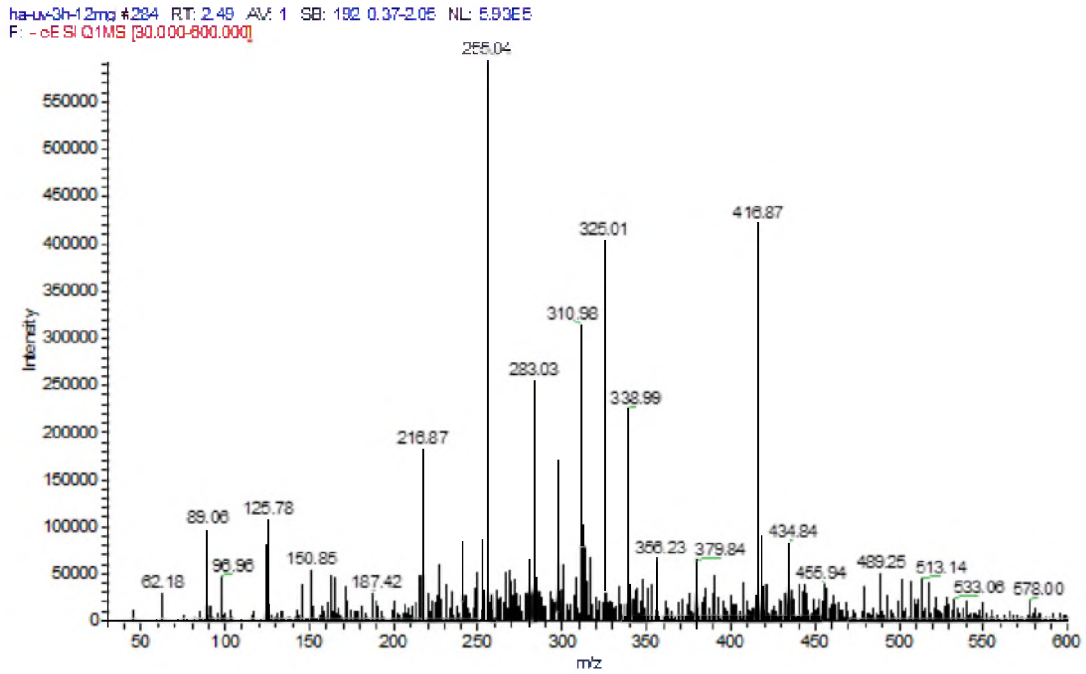

Figure 4.12: Mass spectrum of UV-treated sample containing HS

For TCS, indirect photolysis (with TOC concentration of 5 mg/L) results in more degradation compared to direct photolysis conducted in pure water. Therefore, the EC_{50} value should be higher in indirect photolysis if TCS was the only toxic compound and the photodegradation products did not exert any toxicity. However, the bacterial EC_{50} values did not show a significant difference for direct and indirect photolysis suggesting that the indirect photodegradation products exert toxicity to bacteria.

4.7 OUR Results

To determine effect of TCS on both activated sludge process OUR inhibition tests were conducted. OUR tests were performed with biomass that was unacclimatized to TCS. TCS solutions were prepared as 0.8, 1.6 and 6 mg/L respectively and dissolved O2 measurements continued until the whole substrate was consumed. 0.6 and 1.6 mg/L of TCS did not result in significant inhibition in activated sludge (Figure 4.13, Figure 4.14).

However, when 6 mg/L was added to unacclimatized activated sludge, inhibitory effects were observed on maximum growth rate for X_H (μ_H) and half saturation constant for growth of X_H (K_S). OUR profile of the control and 6 mg/L TCS added activated sludge can be seen in the Figure 4.15.

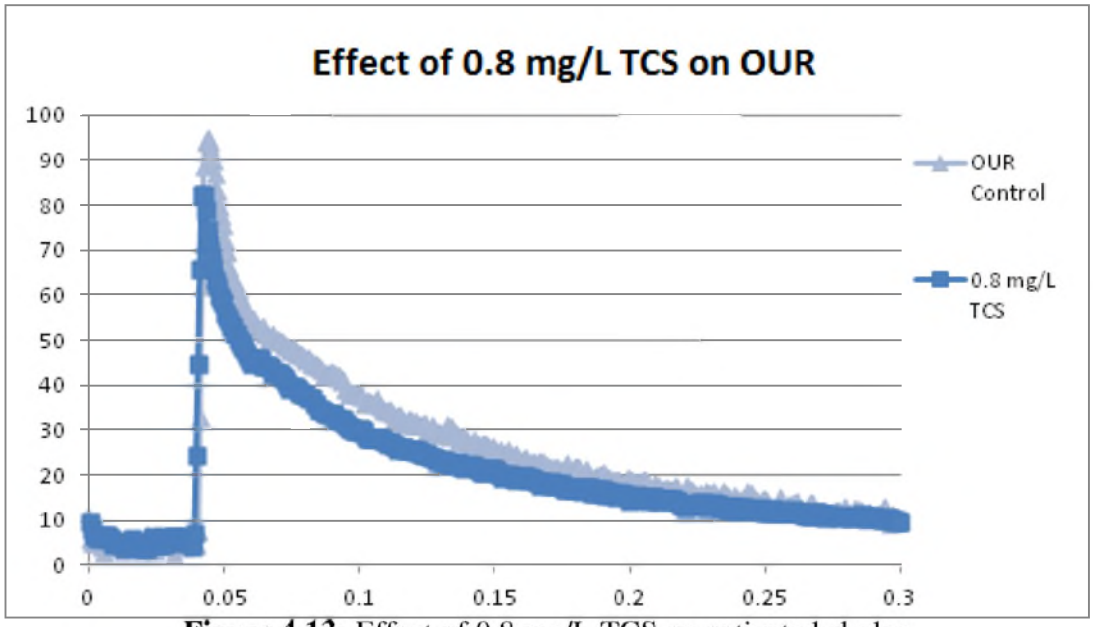

Figure 4.13: Effect of 0.8 mg/L TCS on activated sludge

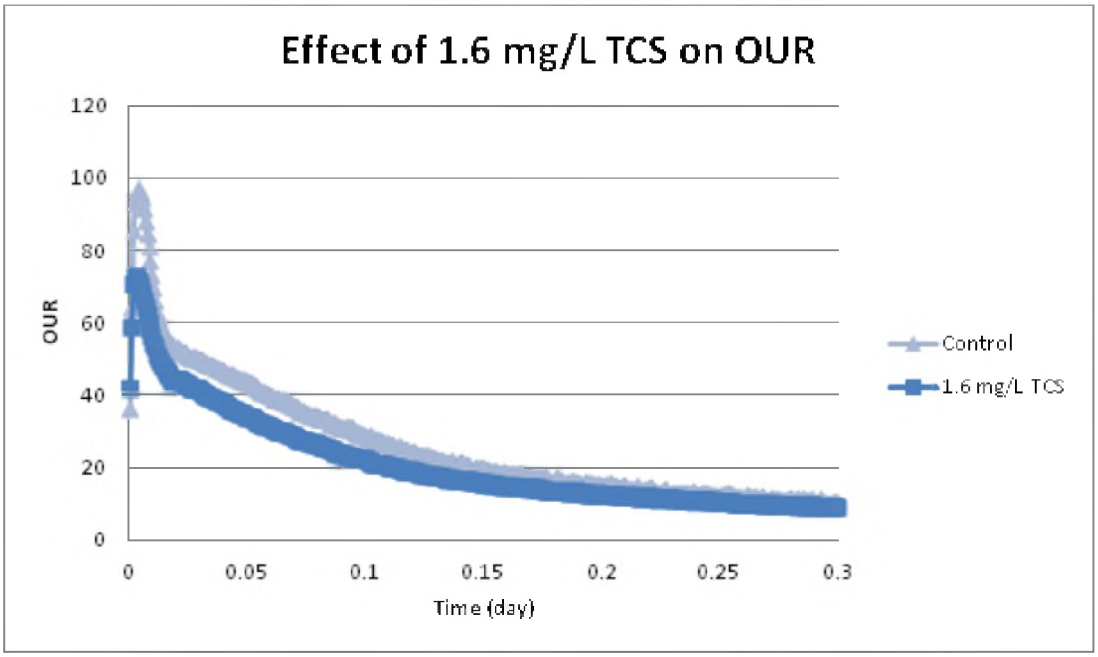

Figure 4.14: Effect of 1.6 mg/L TCS on activated sludge

Effect of 1.6 and 6 mg/L TCS on carbon removal kinetics are summarized in the Table 4.3. Although chemicals may result in competitive, non-competitive and substrate inhibition, the OUR results suggest that 6 mg/L TCS addition led to non-competitive inhibiton since non-competitive inhibition is characterized by its effect on both K_s and μ_H.

Table 4.3: Effect of Triclosan on kinetics of carbon removal

Model Parameter		Unit	Control	1.6mg/L Triclosan Addition	6 mg/L Triclosan Addition
Maximum growth rate for X_H	μ_H	1/day	5.0	5.1	3.3
Half saturation constant for growth of X_H	K_S	mg COD/L	6	7	60
Endogenous decay rate for X_H	b_H	1/day	0.2	0.2	0.2
Maximum hydrolysis rate for S_{H1}	k_h	1/day	3.3	3.3	3.3
Hydrolysis half saturation constant for S_{H1}	K_X	g COD/g COD	0.09	0.09	0.09
Maximum hydrolysis rate for X_{S1}	k_{hx}	1/day	1.10	1.10	1.10
Hydrolysis half saturation constant for X_{S1}	K_{XX}	g COD/g COD	0.09	0.09	0.09
Yield coefficient of X_H	Y_H	g COD/g COD	0.6	0.6	0.6
Fraction of biomass converted to S_P	f_{ES}	-	0.05	0.05	0.05
Fraction of biomass converted to X_P	f_{EX}	-	0.15	0.15	0.15
State variables					
Total biomass	X_{T1}	mgCOD/L	1330	1200	1845
Initial active biomass	X_{H1}	mg COD/L	950	850	1150
Activity		%	71	71	62
Initial amount of biodegradable COD	C_{S1}	mg COD/L	380	300	380
Initial amount of readily biodegradable COD	S_{S1}	mg COD/L	25	20	25
Initial amount of readily hydrolysable COD	S_{H1}	mg COD/L	175	138	175
Initial amount of hydrolysable COD	S_{H2}	mg COD/L	180	142	180

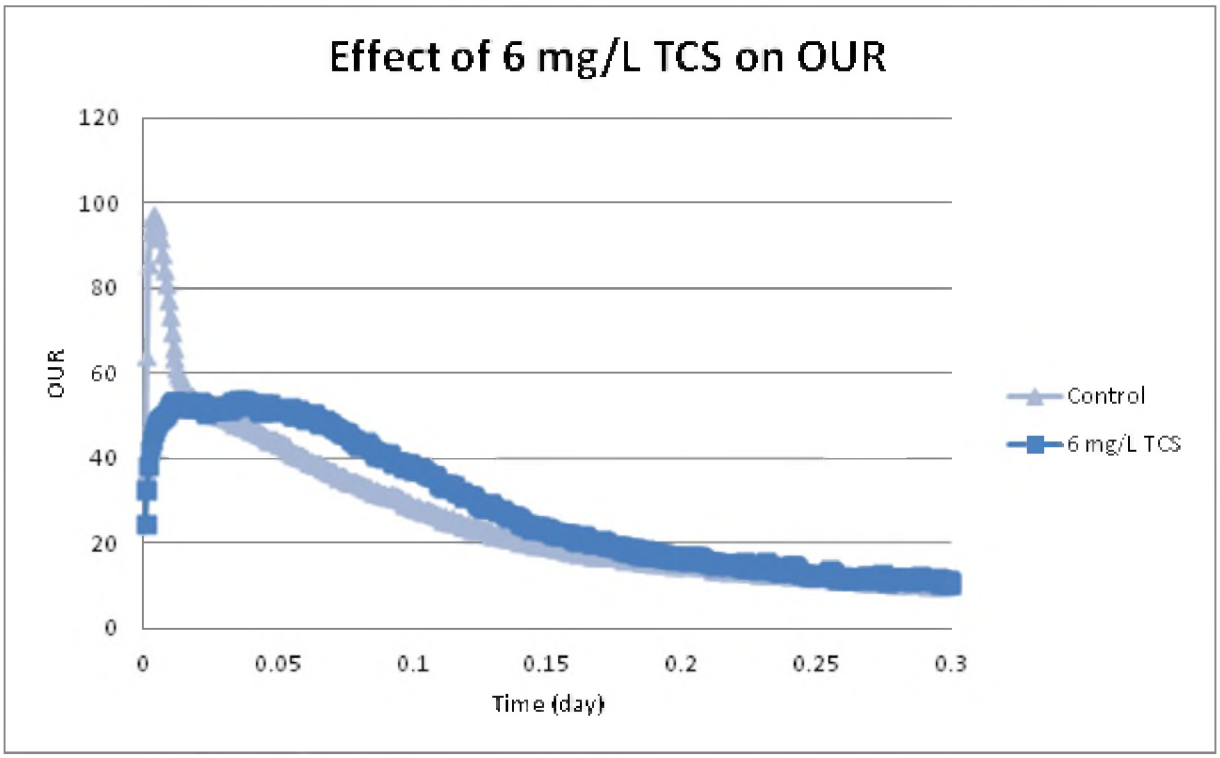

Figure 4.15: Effect of 6 mg/L TCS on activated sludge

5. CONCLUSIONS

The high usage of triclosan in PPCP necessitates studies on its effect on aquatic organisms since most of it will be discharged to wastewaters., The occurrence of TCS in wastewaters has been reported to range mostly between 0.5-30 μg/L even though higher concentrations such as 0.6 mg/L has also been reported. In this study, the possible toxic effects of TCS in freshwater and seawater systems were examined by using two pure cultures (*V. fischeri* and *P. subcapitata*) and a mixed culture (activated sludge). Additionally, effects of direct and indirect photolysis on TCS' toxicity were also examined to provide insight into its effect under real conditions.

The possibility of inhibition of TCS to activated sludge systems were studied using three concentrations of 0.8 mg/L, 1.6 and 6 mg/L. The presence of TCS is not expected to affect the activated sludge process since typical influent concentrations of TCS are below 1.6 mg/L at which no inhibition effect to activated sludge biomass was observed in this study. Besides, in case of possible shock loading of approximately 6 mg/L of TCS, non-competitive inhibition on activated sludge biomass would be observed, but the process is not expected to be significantly affected due to its mixed culture characteristics.

On the other hand, the effect of TCS is expected to be more significant on pure cultures of both bacteria and algae. Indeed, the EC_{50} values were as found as 0.67 mg/L and 7.74 μg/L for bioluminescent marine bacteria *V. fischeri* and freshwater algae *P. subcapitata*. These results indicate that algal species are 90 times more sensitive to TCS compared with the *V. fischeri*.

Upon the discharge of TCS into receiving environments such as lakes and rivers, several process such as adsorption or photolysis will affect its fate and hence its toxicity to the aquatic organisms. The highly moderate K_{ow} (4.8) of the compound suggests that its toxicity may be reduced when it is present in turbid waters containing high concentrations of suspended solids or sediments. On the other hand, photolysis will be the process of importance in clear waters in temperate or tropical areas. The exposure

of TCS to UV light decreased the toxicity to both *V. fischeri* and *P. subcapitata* as indicated by the increase in EC_{50} values. The EC_{50} for direct photolysis of TCS were found as 1.29 mg/L and 8.70 μg/L for *V. fischeri* and *P. Subcapitata* respectively. The significant decrease in toxicity is due to the decrease in the concentration of TCS which was confirmed with LC-MS/MS measurements where 40% decrease in the signal intensity of TCS was observed.

Although direct photolysis is significant for solutions containing no natural organic matter, the other type of photolysis, namely, indirect photolysis may also be significant in natural waters containing humic substances. Hence, the effect of photolysis on the toxicity of TCS on bacteria was further studied using solutions containing known concentrations of humic substances. Indirect photolysis proved to be more effective in reducing the toxicity with the EC_{50} value increasing to 1.37 mg/L. On the other hand, when the change in the intensity of the TCS peaks are compared between the raw water and direct photolysis as well as raw water and indirect photolysis, the decrease in the concentration of TCS would be expected to result in a even higher EC_{50} value for indirect photolysis. Therefore, it is possible that the photodegradation products such as 2,8 DCDD and 2,4 DCP that may form during indirect photolysis themselves exert some toxicity on the bacteria. The presence of 2,4 DCP has not been confirmed as a photodegradation product in our study, but more research with controlled pH values will be useful for the analysis of photodegradation products.

The results obtained during this study suggest that the presence of TCS in wastewater influents and receiving waters are not expected to cause significant acute toxicity on pure or mixed cultures of bacteria. Similarly, although algal species seem to be more susceptible to the toxic effects of TCS, the obtained EC_{50} values are not in the range of TCS concentrations observed in natural waters, Nevertheless, one of the most significant findings of this study is that the EC_{10} value for photolyzed TCS represents concentrations that are commonly found in surface water. Since the with EC_{10} values represent the lowest observed effect level, this finding is important for the health of the aquatic community. In addition, although the EC_{50} values for *P. subcapitata* increase upon photolysis, the EC_{10} value for the photolyzed TCS is smaller than the unphotolyzed TCS (0.6 vs. 3.5 μg/L), indicating the possible increase in toxicity due to photolysis at low concentrations.

In addition to its acute toxic effects, there are several other concerns due to the presence of TCS in the aquatic environments. The first concern is the acute toxicity of TCS when present in mixtures. Since TCS is not expected to occur as a single compound in natural waters, but rather along with other PPCPs, a higher toxicity might be observed if there is an synergistic effect of these PPCP. The second concern is the bioaccumulation of TCS in higher organisms such as invertebrates and it needs to be further studied. The third concern is about the possibility of formation of bacterial resistance due to the exposure to low concentrations of TCS over long periods. The fourth and last concern is the possible endocrine disrupting effect of TCS due to its structure. Studies using assays such as the yeast estrogenicity screen might be useful in detecting such estrogenic effects.

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