TRICLOSAN IN BIOLOGICAL WASTEWATER TREATMENT: FATE, KINETICS AND POPULATION DYNAMICS ASPECTS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

AYBALA KOÇ ORHON

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
ENVIRONMENTAL ENGINEERING

JUNE 2014

Approval of the thesis:

TRICLOSAN IN BIOLOGICAL WASTEWATER TREATMENT: FATE, KINETICS AND POPULATION DYNAMICS ASPECTS

submitted by **AYBALA KOÇ ORHON** in partial fulfillment of the requirements for the degree of **Master of Science in Environmental Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. F. Dilek Sanin _____
Head of Department, **Environmental Engineering**

Prof. Dr. Ülkü Yetiş _____
Supervisor, **Environmental Engineering Dept., METU**

Prof. Dr. Filiz B. Dilek _____
Co-Supervisor, **Environmental Engineering Dept., METU**

Examining Committee Members:

Assist. Prof. Dr. Tuba H. Ergüder _____
Environmental Engineering Dept., METU

Prof. Dr. Ülkü Yetiş _____
Environmental Engineering Dept., METU

Prof. Dr. Filiz B. Dilek _____
Environmental Engineering Dept., METU

Assoc. Prof. Dr. Erkan Şahinkaya _____
Bioengineering Dept., Istanbul Medeniyet University

Dr. Yakup Karaaslan _____
Deputy Director General of Water Management, MoFWA

Date: _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Aybala Koç Orhon

Signature :

ABSTRACT

TRICLOSAN IN BIOLOGICAL WASTEWATER TREATMENT: FATE, KINETICS AND POPULATION DYNAMICS ASPECTS

Koç Orhon, Aybala
M.Sc., Department of Environmental Engineering
Supervisor: Prof. Dr. Ülkü Yetiş
Co-Supervisor: Prof. Dr. Filiz B. Dilek

June 2014, 140 pages

Biocides like Triclosan (TCS) have been detected in several surface waters due to common usage and following wastewater discharges. This situation necessitates detailed researches on accumulation of these compounds in environment, their effects and removal in treatment systems. In this study, a detailed investigation on biological treatability of TCS bearing wastewaters (100 ng/L-100 mg/L) was conducted. A laboratory-scale activated sludge unit was operated for TCS-acclimated and non-acclimated cultures. Fate of TCS was also studied by constructing mass balances. Possible by-product formation was monitored, as well. It was observed that COD removal declined with increase in TCS, however, COD removals were higher with acclimated cultures than with non-nacclimated ones. Adsorption of TCS onto biomass was evidenced with increased proportions at higher TCS concentrations. 2,4-dichlorophenol and 2,4-dichloroanisole were detected as by-products of TCS biodegradation and acclimation resulted in lower by-product levels. Batch kinetic analysis demonstrated that TCS did not interfere with utilization of biodegradable substrate as long as it was kept below 20 mg/L. Presence of TCS resulted in microbial metabolism to shift

from biosynthesis to energy metabolism. Moreover, effect of TCS on microbial population of sludge was searched through molecular biological techniques. In total, 106 microbial species were detected. Although it could not be possible to draw a concrete conclusion and relate them to biokinetic findings due to having too many common species and no information on their dominancy, it was evident that culture compositions vary with acclimation which appears as an important factor for TCS removal, kinetics and by-product formation.

Keywords: Triclosan, activated sludge, COD removal, acclimation, mass balance.

ÖZ

BİYOLOJİK ATIKSU ARITIMI İLE TRİKLOSAN GİDERİMİ: AKIBET, KİNETİK VE POPÜLASYON DİNAMİĞİ

Koç Orhon, Aybala
Yüksek Lisans, Çevre Mühendisliği Bölümü
Tez Yöneticisi: Prof. Dr. Ülkü Yetiş
Ortak Tez Yöneticisi: Prof. Dr. Filiz B. Dilek

Haziran 2014, 140 sayfa

Yoğun kullanımları ve beraberindeki atıksu deşarjları ile Triklosan (TCS) gibi biyositlere yüzeysel su kaynaklarında rastlanmaktadır. Bu durum, söz konusu biyositlerin çevrede birikimi, etkisi ve arıtma sistemlerinde giderimi üzerine detaylı araştırmalar yapılması ihtiyacını doğurmuştur. Bu çalışmada TCS içeren atıksuların (100 ng/L-100 mg/L) biyolojik arıtılabilirliğine ilişkin kapsamlı bir çalışma yapılması hedeflenmiş olup, laboratuvar ölçekli aktif çamur reaktörleri TCS'ye aklime olmuş ve olmamış kültürler ile işletilmiştir. Kütle dengesi kurularak TCS'nin akıbeti üzerine çalışmalar yapılmış, muhtemel yan ürün oluşumu izlenmiştir. KOİ gideriminin artan TCS konsantrasyonları ile azaldığı, ancak mikrobiyal kültürün TCS'ye adapte edildiği koşullarda, adapte edilmediği koşullara göre, KOİ gideriminin daha yüksek olduğu tespit edilmiştir. TCS'nin bir kısmının çamura adsorplandığı ve adsorplanma yüzdesinin TCS konsantrasyonu ile arttığı görülmüştür. TCS nin biyolojik olarak parçalanma sonrası yan ürün olarak 2,4-diklorofenol ve 2,4-dikloroanisol'e dönüştüğü ve konsantrasyonlarının aklimasyon ile azaldığı gözlenmiştir. Kesikli reaktörlerde yapılan biyokinetik çalışmalar, kolay parçalanabilir substrat

varlığında, TCS konsantrasyonun 1-20 mg/L arasında olduğu koşullarda, pepton kullanımını etkilemediği; 20 mg/L'nin üzerinde olumsuz yönde etkilediğini göstermiştir. TCS varlığı mikrobiyal metabolizmayı biyosentezden enerji metabolizmasına yöneltmiştir. Ayrıca, TCS'nin mikrobiyal topluluk üzerine etkisi incelenmiş ve toplam 106 tür tespit edilmiştir. Aklime ve aklime olmamış kültürlerde ortak tür sayısının fazla olması ve türlerin baskınlık durumunun bilinmiyor olması nedeniyle, etkin türler hakkında net bir sonuca varmak ve kinetik bulgularla doğrudan ilişkilendirmek mümkün olamasa da, kültürün TCS'ye aklimasyonuna bağlı olarak değişebildiği ve bu durumun TCS arıtım düzeyi, kinetiği ve yan ürün oluşumunda önemli bir faktör olduğu anlaşılmıştır.

Anahtar Kelimeler: Triklosan, aktif çamur, KOİ giderimi, aklimasyon, kütle dengesi.

To My Family and Lovely Husband

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Prof. Dr. Ülkü Yetiş and my co-supervisor Prof. Dr. Filiz B. Dilek for their invaluable support, patience, encouragement, guidance and insights throughout my study.

I would like to express my sincere thanks to Assoc. Prof. Dr. Erkan Şahinkaya and Research Assistant Beste Çalımlıoğlu for their valuable support and knowledge sharing throughout my study.

I would like to express my gratitude to Supervising Committee Members for their valuable appreciations and contributions to my study.

This study was supported by a project of the Scientific and Technological Research Council of Turkey (TUBITAK – 1112Y210); I also want to thank them for the support.

Many thanks to my colleagues from the Ministry of Forestry and Water Affairs for their friendship, encouragements and positive energy during my study.

I wish to express my gratitude to my mother Raziye Koç, my father Orhan Koç and my brother Baran Koç for their confidence, understanding and belief in me. I appreciate their support and love throughout my life.

Last but not least, I would like to express my warmest thanks and love to my dearie husband Kemal Berk Orhon for his endless moral and technical support and encouragement. I could have never completed this thesis without him.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

ABBREVIATIONS

BLAST : Basic Local Alignment Search Tool

COD : Chemical Oxygen Demand

2,4-DCA : 2,4-dichloroanisole

2,4-DCP : 2,4-dichlorophenol

DGGE : Denaturing Gradient Gel Electrophoresis

EC_{50} : Half Maximal Effective Concentration

EU : European Union

HPLC : High Performance Liquid Chromatography

LOD : Limit of Detection

MLSS : Mixed Liquid Suspended Solids

MLVSS : Mixed Liquid Volatile Suspended Solids

PCR : Polymerase Chain Reaction

PNEC : Predicted No Effect Concentration

SCCP : Scientific Committee on Consumer Products

SVI : Sludge Volume Index

TCS : Triclosan

WTP : Water Treatment Plant

WWTP : Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION

The Biocidal Products Directive of the European Parliament and of the Council (Directive 98/8/EC) has defined the biocidal products as "active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means" (Article 2). Biocides have been utilised in a broad spectrum for various purposes. The major usage areas include cosmetic, detergent, dye, pharmaceutical, plastic, textile, printing inks and wood processing industry.

Triclosan (TCS) (5-chloro-2-[2,4-dichlorophenoxy]-phenol) is one of the most commonly used antimicrobial biocides in the daily life and it exists as an ingredient in the several pharmaceutical and personal care products such as soaps, toothpaste, shave gels, deodorants and detergents (Dann and Hontela, 2011). There are some remarks on that its usage in the cosmetic products up to a certain level does not constitute any problem. For instance, European Union (EU) Scientific Committee on Consumer Products (SSCP) (2010) has reported that TCS plays an important role on the prevention against the infections when it is used in suitable amounts in the cosmetic products.

At the same time, as laid down in Annex VI and Part I, EU Council Directive on the approximation of the laws of the Member States relating to cosmetic products (Directive 76/768/EEC) approves the use of TCS in cosmetic products as a preservative in order to inhibit the development of

microorganism when it is added up to a percentage (maximum authorized concentration) of 0.3% in such consumer products. However, TCS has been encountered in various concentrations in the numerous environmental media due to its usage at different amounts in different areas. This situation brings about a need for the scientific studies that will be on the investigation of the potential effects associated with TCS presence in environmental media and removal of this biocide from these media.

Recently, there have been conducted lots of studies regarding the removal of TCS and other biocides from both the domestic wastewater treatment plants (WWTP) and drinking water treatment plants (WTP). According to these studies, different removal efficiencies have been reported and adsorption has been stated to be the important mechanism for the removal of the substantial part of TCS in activated sludge systems due to its having a chlorinated structure (Singer et al., 2002; Bester, 2003; Heidler and Halden, 2007).

TCS removal efficiency of the WWTPs varies with the treatment process involved in the plant and hydraulic retention time through the plant. Ozone treatment, activated carbon, membrane filtration, phenton oxidation and activated sludge systems are the most known treatment technologies for TCS.

It is a known fact that TCS might be transformed into other potential toxic compounds (i.e. dioxins, chloroform, 2,4-dichlorophenol, 2,4,6-trichlorophenol and other chlorinated organics) by means of the photodegradation or biodegradation right after discharge to the environment (NICNAS, 2009; Orvos, 2002; Chen et al., 2011). Dioxins, one of the transformation products of TCS, are extremely toxic substances even in small amounts. They are also known as endocrine distruptors and their disposal rate from the human body is so slow, and the last one important thing on these substances is that they remain in the environment for a long

time. Furthermore, there exist also scientific studies in the literature indicating the transformation of TCS to more toxic by-products such as chlorinated phenoxy-phenol, chlorinated phenols and trihalomethanes at the end of chlorination process applied for disinfection purposes in the WTPs and WWTPs (Kanetoshi et al., 1987; Rule et al., 2005). In other respects, soil environment might be adversely affected depending upon TCS level it is subjected through the discharge of sewage sludge concentrated with the pollutant due to the process of adsorption in activated sludge systems.

On the other hand, use and subsequent discharge of TCS to the environment results in the development of antibiotic resistance by certain microorganisms, also known as selection for less susceptible microorganisms, and several allergic cases have been observed to be frequently encountered as a result of the increase in number of this kind of resistant bacterias in the environment (Ying and Kookona, 2007; Walsh et al., 2003; Fernández-Fuentes et al., 2012). There is a need for more detailed scientific researches regarding the biological treatment and fate of TCS in WWTPs due to the fact that it has been measured in numerous surface waters (0.16-2300 ng/L) and especially in WWTP effluents (0.1-269 μg/L) with higher concentrations than its actual determined toxicity levels to protect aquatic life in receiving bodies (i.e. 50 ng/L as predicted no effect concentration for surface waters, PNEC; 0.7-620 μg/L as half maximal effective concentration for aquatic organisms, EC_{50}), it has been reported to be seriously accumulated on sludges (0.028-37.2 mg/kg) and it has a potential to transform into more toxic compounds (BIOHYPO, 2010-2012; Kolpin et al., 2002; Mezcua et al., 2004; Svensson, 2002; Ying and Kookana, 2007; US EPA Ecotox Database).

Adaptation of microbial culture to TCS was not taken into consideration within the scope of the earlier studies conducted for the biological treatment of this biocide in WWTPs. Lots of the studies in the literature have indicated that acclimation to the contaminant enhanced the treatment

performance of the biological systems and treatment potential of the microbial cultures increased seriously through the reduction or complete elimination of the compound inhibition effect thanks to prior adaptation (Yetiş et al., 1999; Dilek et al., 1998; Şahinkaya and Dilek, 2006 and 2007; Silvestre et al., 2011; Vaiopoulou and Gikas, 2012). Therefore, in order to fill the gaps in the literature, a comprehensive study was conducted regarding the biological treatability of TCS bearing wastewaters with acclimated and non-acclimated microbial cultures and essential data needed for the optimization of TCS removal in the activated sludge systems were aimed to be obtained within the scope of this thesis study.

In accordance with this aim, in running tests with TCS acclimated culture, a laboratory-scale activated sludge unit (pulse-fed sequencing batch reactor) was continuously operated at a sludge age of 8 d in order to stimulate a real biological WWTP and it was fed with domestic wastewater that was prepared synthetically. TCS content of the synthetic wastewater intermittently and gradually increased to allow for the acclimation of microbial culture and the system was investigated in terms of its removal performance for both chemical oxygen demand (COD) and TCS, mixed liquid suspended solids (MLSS) and mixed liquid volatile suspended solids (MLVSS) concentrations through the biological treatment process and sludge settleability characteristics.

Moreover, amount of TCS (as both mass and concentration) adsorbed to the sludge was determined following the measurements in effluent water and sludge, and mass balance of TCS was constructed for a laboratory-scale biological treatment unit. The fate of TCS was revealed in the system and major mechanisms affecting its fate were clearly introduced. Possible biodegration by-products of TCS were also designated and quantified using the measurements in solid and liquid samples within this study.

On the other hand, DNA extraction, Polymerase Chain Reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing analysis were conducted for the sludge samples taken from the reactor operated at different TCS concentrations in order to investigate the change in dominant microbial species with TCS content of sludges.

Similarly, pulse-fed sequencing batch reactors were also operated with the sludge samples non-acclimated to TCS in order to observe TCS removal in the absence of contaminant acclimation by microorganisms and to comprehend the effect of this situation to the other aforementioned variables in the system.

Furthermore, batch reactor operations were also performed using TCS containing feed wastewaters and the biokinetic behavior of the systems including acclimated and non-acclimated microbial cultures was tried to be understood and compared accordingly.

CHAPTER 2

LITERATURE REVIEW

2.1 Physical and Chemical Properties of Triclosan

TCS is a chlorinated organic compound of white crystalline powder having a broad spectrum of usage area in daily life. It is integrated into the numerous pharmaceutical and personal care products for the antimicrobial purposes (Dann and Hontela, 2011).

The physical and chemical characteristics are essential to be recognized in order to gain a clear understanding on the mechanisms designating the environmental fate of the compound. In this sense, structural formula and physical and chemical properties of TCS were represented in Figure 1 and Table 1, respectively.

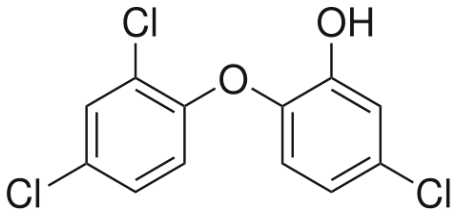


Figure 1 TCS (Allmyr, 2009)

Table 1 Physical and chemical properties of TCS (EU SCCP, 2010; NICNAS, 2009)

Name of Property	**Explanation/Value**
CAS Number	3380-34-5
EC Number	222-182-2

Table 1 Physical and chemical properties of TCS (Cont'd)

Name of Property	**Explanation/Value**
Synonyms	2,4,4'-trichloro-2'-hydroxy-diphenylether; 5-Chloro-2-(2,4-dichlorophenoxy)phenol; Trichloro-2'-hydroxydiphenylether
Chemical Formula	$C_{12}H_7Cl_3O_2$
Form	White crystalline powder
Stability	Stable more than 9 years if stored under normal conditions
Molecular Weight	289.54 g/mole
Density	1.55 ± 0.04 g/cm^3
Dissociation Constant (pK_a)	8.14 at 20 °C
Melting Point	57 ± 1°C
Water Solubility	1 mg/L at 20 °C
Octanol-Water Partition Constant (log K_{ow})	4.76 at 20°C
Vapor pressure	4×10^{-6} mmHg at 20°C

As seen from Table 1, TCS has hydrophobic nature (i.e. higher octanol-water partition coefficient) which at the end causing a lower water solubility. There are studies in the literature stating its tendency to remain in the solid phases and thus adsorption to these phases due to its lower water solubility (Lee and Peart, 2002; NICNAS, 2009).

2.2 Production and Use of Triclosan around the World

Biocides (i.e. TCS, Benzalkoniumchloride and Chlorhexidine) are the chemicals that are added as a preservative or an antiseptic agent to the commonly used products in daily life such as personal care products like soaps and toothpaste, house cleaning agents, sportswears, shoes and textile

products like carpets. Their abundance in the cosmetic products varies between the ranges of 0.1-0.3% by weight (Sabaliunas et al., 2003).

TCS is one of the widely used biocide and it is produced since the year of 1965 by the Ciba Specialty Chemicals Corporation (Levy et al, 1998). There exist numerous studies reporting its production with an amount of 350 tones per year only in Europe (Singer et al., 2002). Considering the amount that is also imported, the consumption in Europe may reach to significant levels. It has been stated that approximately 450 tonnes of TCS was consumed around Europe in the year of 2006 without the information on the contributions of production and imports to this amount of consumption (EU SCCP, 2010). However, the production and consumption amounts reported in different studies indicate that consumption value is more than that of production and the difference can be thought as the amount of TCS entering the Europe thanks to import from other nations.

Expectedly, the consumption of TCS changes with country to country depending on the level of development, economical and social factors, environmental factors, public awareness on the hazardous chemicals, community needs, dominant industrial activities, production processes involved in the industries and so on. For instance, the study of Adolfsson-Erici et al. (2002) demonstrated that 2 tonnes TCS is consumed annually in Sweden whereas Xie et al. (2008) reported annual TCS consumption of around 40 tonnes in Germany, 20 times greater than that of Sweden. On the other hand, TCS consumption value has been stated to be over 300 tonnes per year in USA (Halden and Paull, 2005).

There are also some countries not producing TCS but importing it from different countries. Australia can be a good example to this subject. In Australia, TCS is not commercially produced; however, it enters the country by means of imports either as raw material itself or the active compound in different consumer products (NICNAS, 2009).

Unfortunately, there are no inventory records of annual production, import and consumption of TCS in Turkey. Thus, it is not possible to monitor the annual trends, understand the contribution of each item to the abundance of TCS in the country-based level and comment on the decrease or increase in TCS amounts through these phases.

Main usage areas of TCS include:

- Personal care products (i.e. deodorants, face and skin cleansers, facial masks, body sprays, toothpastes, mouthwashes, hand soaps, shower gels, eye make-up, cotton buds, baby wipes, nail conditioners, colognes)
- Healthcare and medical devices (i.e. ureteral stents, surgical sutures, foley catheter)
- Household and other consuming products (i.e. liquid soaps, bathroom surface cleaning products, detergents, toys, carpets, textiles, fabrics, latex paints, plastics, garbage cans, kitchen utensils, food storage containers)
- Veterinary hygiene products (i.e. pet shampoos, insect repellents, cattle teat ointment)
- Equipments like conveyor belts, fire hoses, dye bath vats and ice maker device
- Swimming pool liners, air conditioning systems, aquariums (EU SCCP, 2010; NICNAS, 2009; US EPA, 2008).

On the other hand, TCS is not allowed to be used as disinfectant and preservative in the food and feed production through the Directives of 98/8/EC and 95/2/EC and Regulation (EC) 1831/2003 (EU SCCP, 2010). Furthermore, Germany introduced a ban on the use of TCS within the plastic products having a direct contact with foods since September 2009 (EU SCCP, 2010). Besides, the use of TCS as a preservative in the cosmetic products has also been restricted to 0.3% in EU by the Directive 76/768/EEC.

When the distribution of TCS consumption among various usage areas in Europe is investigated, it is observed that the great portion with a percentage of around 85% is utilized in personal care products whereas 5% and 10% of it is consumed in textile products and plastics and food contact materials, respectively (EU SCCP, 2010).

2.3 Environmental Fate of Triclosan

2.3.1 Abundance in Environmental Samples

Although TCS is not a naturally found substance in the environment, it has been encountered in several environmental mediums at different concentrations (Government of Canada, 2012). Its existence in environment comes from the anthropogenic activities as indicated above. Its addition to the broad spectrum products as a preservative, disinfectant and odor-controlling agent in higher concentrations and its common usage in the world has resulted in the discharges of wastewaters contaminated with this compound to the WWTPs and right after these TCS including wastewaters have reached to the surface water resources. Therefore, the presence of TCS like biocides in the environmental systems has become a prominent issue in recent years in parallel with the increased use of such products. It has been reported that the detection of TCS in surface water bodies (i.e. lakes, rivers, estuarine waters, marine waters) is thorough the effluents of domestic and industrial WWTPs, urban rain waters and agricultural activities (NICNAS, 2009).

TCS has been also detected in the soil environments as a result of the land application of biosolids after sewage sludge treatment (EU SCCP, 2010). Cha and Cupples (2009) demonstrated that the biosolid samples taken from three WWTPs of Michigan were found to contain TCS with amounts in the range of 90-7060 μg/kg. TCS concentration of 0.16-1.02 μg/kg was

observed in the soil samples gathered from the ten agricultural sites applied with these biosolids.

Despite the limited literature data on the level of presence of the biocides in natural environments, there exist some studies regarding the concentration of one of the widely used biocide, TCS, in environmental systems and WWTPS influents and effluents. TCS levels of the environmental samples from various countries are summarized in Table 2.

Table 2 TCS levels of the environmental samples

TCS	Sample	Source
29000 μg/kg	WWTP sludge- Washington State	US EPA, 2003
900-28200 μg/kg 12500 μg/kg (ort)	WWTP sludges- Canada	Lee and Peart, 2002
28-6400 μg/kg	WWTP sludges- Sweden	Svensson, 2002
2800-4400 μg/kg	WWTP sludges- Sweden	Remberger et al., 2002
99.3 ng/L	Marine water- Victoria Harbor	Wu et al., 2007
108 ng/L	WWTP- Australia	Ying and Kookana, 2007
40 ng/L (ort) 2300 ng/L-max	Surface waters- USA	Kolpin et al., 2002
75 ng/L	Rivers fed by WWTPs- Australia	Ying and Kookana, 2007
18-98 ng/L	Surface waters- Switzerland	Singer et al., 2002
1.4-14 ng/L	Lake Greifensee- Switzerland	Lindstrom et al., 2002
5.58 mg/kg	Biomass- Australia	Ying and Kookana, 2007
1.1-1.3 μg/L	WWTP- Germany	Bester, 2003
30-90 ng/L	Surface waters- Germany	Wind et al., 2004
410 ng/L	WWTP effluent- OH, USA	Hua et al., 2005
<0.4 μg/L	Urban wastewater- Portugal	Silva and Nogueira, 2008

Table 2 TCS levels of the environmental samples (Cont'd)

TCS	**Sample**	**Source**
16.2 ng/L	Marine water- Hong Kong	Wu et al., 2007
26 ng/L	Riverine water- Sha Tin, Hong Kong	Wu et al., 2007
142 ng/L	WWTP effluent- Sha Tin, Hong Kong	Wu et al., 2007
0.16-757.7 ng/L	Surface waters- Ankara, Turkey	BIOHYPO, 2010-2012
< 0.87-11.44 ng/L	Wastewater- Ankara, Turkey	BIOHYPO, 2010-2012
16289-37189 µg/kg	WWTP sludges- Ankara, Turkey	BIOHYPO, 2010-2012
0.65-11.15 ng/L	Çamlıdere Reservoir-Ankara, Turkey	Yavuz, 2013
<0.86-48.96 ng/L	Çamlıdere Reservoir-Ankara, Turkey	Yavuz, 2013
<0.95-757.7 ng/L	Eymir Lake- Ankara,Turkey	Yavuz, 2013
9.07 µg/L	Hospital WWTP influent-Greece	Gatidou et al., 2007
1.12 µg/L	Hospital WWTP effluent-Greece	Gatidou et al,. 2007
2.3-562 µg/L	WWTP influent- Spain	Mezcua et al., 2004
0.1-269 µg/L	WWTP effluent- Spain	Mezcua et al., 2004

As can be seen from Table 2, TCS concentration varies between 0.16-2300 ng/L in surface waters, 16.2-99.3 ng/L in marine waters, <0.87-562000 ng/L in wastewaters, 100-269000 ng/L in WWTPs effluents and 28-37189 µg/kg in WWTPs sludges. These values indicate the possibility of TCS observation in the environmental systems within the wide concentration range.

2.3.2 Mechanisms Affecting the Fate

Since TCS has been encountered in several environmental systems, it is crucial to investigate the behavior and fate of TCS in the environment when it is entered to these systems. The behavior and fate of the compound are completely dependent on its physical and chemical properties. Therefore, the mechanisms involved in its environmental fate are summarized in below sections.

2.3.2.1 Hydrolysis

TCS has low water solubility and hydrolysis is not anticipated to be a prominent mechanism for the fate of TCS in the environment (EU SCCP, 2010). TCS has been reported to be hydrolytically stable in water environment no matter of the pH (i.e.especially pH 4, 7 and 9) is and the half life of it in water is over 365 days in the conditions where the temperature is 25°C and pH of the water is between 4 and 9 (Ciba-Geigy Limited, 1990f). Furthermore, the study conducted by Singer et al. (2002) demonstrated that it is still hydrolytically stable under the presence of even strong acids and bases in water resources. This situation was also supported by another study that TCS was not hydrolysed in 15 h when it was remained in a 3 N alcoholic sulphuric acid solution and only the percentage less than <0.5%, a completely ignorable amount, hydrolysed in a solution including 5N caustic soda (CIBA, 1998).

2.3.2.2 Volatilization

Vapor pressure (i.e. 4×10^{-6} mmHg) and Henry's Law constant (i.e. $\approx 10^{-2}$ Pa m^3/mole) of TCS are very low at the temperature range between 20°C and 25°C that it is not expected to volatilize from the water surface (Ciba-Geigy Limited, 1990d). Therefore, air partitioning of TCS is not considered as an important mechanism for its fate in the environment and air concentration of

this compound is pretty low compared to the concentrations observed in other environmental phases (NICNAS, 2009). Furthermore, the predicted half-life of TCS in the atmosphere is relatively short (i.e. 7.96 h) that it is not long-range transported in the air although some portion of it volatilizes from the water surface (NICNAS, 2009)

2.3.2.3 Photolysis

Unlike hydrolysis and volatilization, photolysis is the prominent mechnanism for the well-understanding of TCS fate in the environment and TCS is rapidly degraded by means of photodegradation (EU SCCP, 2010). TCS has been reported to be readily photodegradable when it is continuously irradiated with artificial light at 25°C in water environment with pH 7 and under these conditions; its photolytic half-life in the aqueous medium was observed as 41 min (US EPA, 2008). Furthermore, it was stated again with the US EPA (2008) on TCS that photodegradation half-life up to 10 d may be introduced in lake waters.

Photodegradation of TCS in the environment depends upon the numerous factors such as the availability of sunlight and pH of water (Tixier et al., 2002; Arnold et al., 2003). There also exists information in the literature that the observed photolytic half lifes at pH 6 are 19 times greater than that obtained at pH over 10 in water (NICNAS, 2009).

Another study conducted by Tixier et al. (2002) demonstrated that 80% of TCS removal from a lake water in Switzerland was due to the photodegradation in summer time under laboratory conditions which directly influenced by the sunlight availability. Lindstrom et al. (2002) studied the photolysis of TCS in lake water under natural sunlight conditions by altering the pH of water and they found that TCS was more easily photodegraded at alkaline conditions.

Other issue related with the photodegradation of TCS is the formation of different phototransformation products (i.e. dioxins, chloroform, 2,4-dichlorophenol, 2,4,6-trichlorophenol and other chlorinated organics) which may be more toxic, bioaccumulative and persistent than the parent molecule (NICNAS, 2009; Orvos, 2002; Chen et al., 2011). Possible phototransformation products of TCS are reported to be 2-4-dichlorophenol, 2,7-dichlorodibenzo-p-dioxin, 2,8-dichlorodibenzo-p-dioxin, dichlorohydroxydibenzofuran, 4-chlorocatechol, 4-chloro-2,4'-dihydroxydiphenyl ether, 2,4'-dichloro-2',4-dihydroxydiphenyl ether; 2,4-dichloro-2'-dihydroxydiphenyl ether, 4,4'-dichloro-2-hydroxydiphenyl ether and 2',4-dichloro- 2-hydroxydiphenyl ether (Arnold et al., 2003; Ferrer et al., 2004; Kanetoshi et al., 1987; Latch et al; 2005; Mezcua et al., 2004).

2.3.2.4 Biodegradation

Biodegradation is one of the known fate process of TCS in the environment and it becomes important especially in the aerobic aquatic systems since TCS is accepted to be inherently biodegradable by the microorganisms under this circumstances. Thus, following the enterence to the water bodies, biodegradation contributes to the significant TCS loss from the water phase when the aerobic conditions are prevailing in the system (NICNAS, 2009). This issue was supported with the study conducted by Hansveit and Hamwijk (2003) and they showed that 70% TCS removal from river water was achieved in 28 d by means of biodegradation in the system.

Several metabolites are formed as biodegradation by-products of TCS in water and one of the well known transformation product is the methyl triclosan, methylated form of it, as well. Although, methyl triclosan is more persistent and bioaccumulative compared to TCS, observed concentrations of this product in the receiving waters are substantially lower than those of TCS. Apart from the biodegradation metabolites, TCS may turn to bound

residues and carbondioxide as a consequence of biodegradation and they should be considered in the fate processes (NICNAS, 2009).

As opposed to the aerobic environmental conditions, TCS is not biodegradable under anaerobic conditions due to its persistency in these circumstances and biodegradation is not a considerable fate mechanism affecting its concentration in the receiving bodies (McAvoy et al., 2009; NICNAS, 2009). Under anaerobic conditions, TCS removal around 10% was reported in the one other study where TCS concentration was 200 µg/L and the duration of the monitoring was 147 d (Springborn Laboratories Inc., 1994a).

In the literature, it is stated that TCS concentration in the system inversely affects the rate of its biodegradation in the aquatic environment which is resulting with the greater inhibition of the microbial population when more TCS exists in their habitat. This adverse effect is eliminated under a certain acclimation period of microorganisms to TCS and the rate of biodegradation then becomes independent from the contaminant concentration (NICNAS, 2009). In accordance with this state, Federle et al. (2002) conducted a research in order to see the effect of microbial acclimation on the biodegradability of TCS and monitored the biodegradation with 3 samples; two were adapted to the 200 µg/L TCS and fed with 1 mg/L TCS and the third one was the control. At the end of the study, they demonstrated that biodegradation occurred more rapidly and more TCS was removed by the acclimated microbial population compared to the unadapted ones.

2.3.2.5 Adsorption

Adsorption is one of the mechanism affecting the fate of TCS in the environment since it is a hydrophobic substance with log K_{ow} value of 4.76 and thus, it tends to be adsorbed to the suspended solids and sediments in water bodies which at the end becoming a potential risk for aquatic

organisms (EU SCCP, 2010). This is supported with another study conducted by Mensink et al. (1995) which demonstrating the high affinity of TCS for the sediments rich in organic matters and the low mobility following the adsorption; however, if the sediments are saturated with TCS, there may be increases in the mobility of the compound and it may tend to move on to the liquid phase.

2.4 Reasons to Treat Triclosan

Existence of TCS in the water and sludge samples has accelerated the researches conducted on the adverse impacts of this compound on the surrounding environment. Within the scope of Water Framework Directive (2000/60/EC), TCS has been determined as "specific pollutant" in 3 countries namely Norway, Sweden and Switzerland and therefore the recent studies have focused on this biocide (Republic of Turkey Ministry of Forestry and Water Affairs, 2013). TCS has adverse effects on the organisms living in the environmental systems. It is a known fact that some structural and biochemical variations have been observed on the pure cultures of bacteria, algae, fungi, yeast and protozoa when they are exposed to the antimicrobial biocides (Orvos et al., 2002; NICNAS, 2009). In accordance with this fact, Wilson et al. (2003) reported that algaes in riverine ecosystem may undergo changes in their structural and functional units when they are faced with the effluent discharges from the WWTPs.

The major remark on biocides is for their toxic effects on the certain algae species like *Scenedesmus subspicatus* (Orvos et al., 2002; NICNAS, 2009). For this algae specie, it was found that TCS concentration of 500 ng/L does not result in any adverse effect and when the general accepted safety factor of 10 was taken into consideration, PNEC of TCS on this specie was calculated as 50 ng/L (CIBA, 1998; NICNAS, 2009). However, this value was reported as 690 ng/L by Orvos et al. (2002). Moreover, algae were stated to be the most sensitive aquatic organisms to TCS and their growth

rates were inhibited with the EC_{50} value between the 0.7-19 μg/L (NICNAS, 2009; Orvos et al., 2002).

At the same time, bioaccumulation on the organisms due to its hydrophobic nature is another issue associated with the presence of TCS in the water environment (NICNAS, 2009). Samsoe-Peterson et al. (2003) demonstrated the bioaccumulation of TCS on the fish tissues with 3700 and 8400 bioconcentration factors. This situation implies that even if WWTPs effluents have a low TCS content, it may reach to the toxic levels following the bioaccumulation on the tissues of the environmental organisms.

There are also scientific researches on the endocrine distruption effects of TCS. It was stated that TCS has endocrine distruption effects in amphibians when it is present at current environmentally observed concentrations. Fishes and mammals are the other organisms for which endocrine distruption effects observed at the contaminant concentration much higher than its actual values in the environmental systems (Government of Canada, 2012).

Moreover, soil environment can be adversely affected from TCS through the land application of biosolids and the degree of the effects is definitely based upon the existence level of the contaminant in these biosolids (EU SCCP, 2010). This subject was studied by Ying and Kookona (2007) and they demonstrated that soil amended with the sludges bearing 0.09-16.79 mg/kg (i.e. median concentration of 2.32 mg/kg on dry weight basis) TCS, actual TCS content of sludges in Australia, may adversely be affected with this contaminant. It was also reported in the literature that microbial populations of the receiving soils may be distrupted by the land application of TCS involving biosolids and the irrigation with the effluents of WWTPs; however, soil respiration and nitrification are not affected from this phenomenon (NICNAS, 2009).

Apart from the negative effects mentioned so far, the contamination of the environment with TCS like biocides might lead to the development of antibiotic resistance of bacteria and therefore, recent studies have risen the speculations on the regard that TCS can promote the cross or co-resistance against the antibiotics (Gaze et al., 2005). As a consequence, resistant bacteria may increase in the environment and there may be corresponding increases in the allergic diseases. Considering these effects together with the horizontal (lateral) gene transfer, biocides induced antibiotic resistance is considered to reach serious levels (Ying and Kookona, 2007; Walsh et al., 2003; Fernandez-Fuentes et al., 2012).

Effects of TCS on human healths are the other research area in the literature and it was reported that TCS may lead to the skin, eye and respiratory irritations, allergy susceptibility and inhalation toxicity on human beings (NICNAS, 2009). The further studies are ongoing regarding the neurotoxic, carcinogenic and mutagenic effects of TCS on human health.

At this stage, TCS can be regarded as a threat for the environment and especially for the human beings consuming or having a contact with waters of the receiving bodies. Hence, EU banned the use of TCS in any products having a possibility of direct contact with the foods in March, 2010 (APUA, 2011). That is why the recent studies have canalized on the treatment of such biocides from the water environment through different treatment techniques.

2.5 Treatment Alternatives for Triclosan

2.5.1 Chlorination

Chlorination is commonly applied in the municipal and other WWTPs for the disinfection purposes (NICNAS, 2009). Rule et al. (2005) stated that high TCS removal efficiencies can be achieved by means of free chlorine

treatment of TCS containing wastewaters. During the removal of TCS in this way, Canosa et al. (2005) reported the main pathways of the degradation as the chlorination of the phenolic ring in the parent molecule and cleavage of the ether bond.

In the studies conducted by Kanetoshi et al. (1987) and Rule et al. (2005), it was demonstrated that chlorination of the waters and wastewaters from WWTP resulted in the transformation of TCS to more toxic products like chlorinated phenoxy-phenol, chlorinated phenols and trihalomethanes. 5,6-dichloro-2-(2,4-dichlorophenoy)phenol, 4,5-dichloro-2-(2,4-dichlorophenoxy)phenol), 4,5,6-trichloro-(2,4-dichlorophenoxy)phenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol and chloroform are the substances formed as chlorination metabolites of TCS. US EPA stated 2,4,6-trichlorophenol and chloroform to be the probable human carcinogens based on sufficient evidence of carcinogenity in animals and 2,4-dichlorophenol to be among the Contaminant Candidate List (US EPA IRIS Database).

Another study conducted by Fiss et al. (2007) also demonstrated the formation of the (chlorophenoxy) phenols, 2,4-dichlorophenol, 2,4,6-trichlorophenol and chloroform at the end of chlorination of TCS bearing antimicrobial products as soaps in tap water at pH 7 and they observed that the produced concentration of these by-products was entirely affected with the type of antimicrobial product studied, the free chlorine to TCS ratio in tap water and the temperature of the surrounding environment.

2.5.2 Ozone Treatment

Ozone treatment was reported to be the effective way of treatment for the degradation of organic contaminants such as pharmaceuticals, pesticides and surfactants (Ikehata et al., 2006). In his book, Oppenlander (2003) stated the major advantages of ozone treatment as its ability to achieve the complete mineralization of organic pollutants, the production of the by-products less

important in terms of the possible harms and biodegradability characteristics in the environment, and its durability against the changes in the flow rates and in the composition of the wastewater. The main mechanims involved in the ozone treatment were identified as the ozone attack to the double bonds, activated aromatic rings and neutral amines (Ikehata et al., 2006).

Ozone treatment of the waters and wastewaters contaminated with pharmaceuticals and other micropollutants is the trend topic among the environmental researches in recent years. Suarez et al. (2007) demonstrated that around 100% TCS removal from the municipal wastewater having a 7.5 mg/L dissolved organic content was accomplished thanks to aqueous ozone treatment performed with 4 mg/L ozone. They also reported in this study that TCS removal was variable with the dissolved organic content of the wastewater and it decreased down to 58% for the municipal wastewater with 12.4 mg/L dissolved organics although the aqueous ozone dosage was set to 6 mg/L.

The ozone degradation products of TCS are not well known (EU SCCP, 2010); however, four different ozonation products of TCS was listed as 2,4-dichlorophenol, chlorocatechol, monohydroxy-triclosan and dihydroxy-triclosan in the recent study conducted by Chen et al. (2012).

2.5.3 Membrane Filtration

Membrane filtration processes like nanofiltration and reverse osmosis became very popular and started to be widely used in order to treat trace organic contaminants from the water environment (Agenson et al., 2003).

Dolar and Kosutic (2013) demonstrated that ultrafiltration was less effective for the removal of pharmecuticals from the water bodies (i.e. <50%) compared to the nanofiltration and reverse osmosis (i.e. >90%) due to its having larger molecular weight cutoff. In another study conducted by Kim

et al. (2007), higher TCS removal efficiencies (i.e. >98%) were reported in the wastewaters after the treatment with nanofiltration and reverse osmosis, and membrane filtaration was stated to be the effective way of treatment of micropollutants.

The adsorption of TCS to the membrane surface was another topic of study in that area (Ogutverici, 2013). In his study, Ogutverici (2013) found that greatest part of TCS removal during the nanofiltration occurred due to the adsorption of the hydrophobic compound to the experimental set-up and membrane itself, and thus the higher removal efficiencies reported in the literature do not reflect the truth if the adsorption phenomena was not considered. Ogutverici further stated that around 60-70% TCS removal was achieved by nanofiltration considering the system adsorption.

2.5.4 Biological Treatment

Several biological treatment processes are used around the world in order to remove TCS from the municipal or industrial wastewater streams (Aufiero et al., 2012). These biological treatment systems can be either attached (i.e. trickling filter, rotating biological contactor) or suspended growth process (i.e. conventional activated sludge process, sequencing batch reactors and so on). These two differs based upon the setting of the microorganisms in the system as attached to the surface they grow or suspended to the wastewater during biological treatment (Tchobanoglous et al., 2004).

Thompson et al. (2005) studied the biological treatment of TCS containing wastewaters with rotating biological contactors, trickling filters and activated sludge systems. In this study, they found that activated sludge system was the treatment system giving the higher TCS removal efficiency (i.e. 95-98%) among these different biological treatment systems (i.e. 58-96% in rotating biological contactors and 86-97% in trickling filters). There are several studies for the biological treatment of TCS bearing wastewaters

in the literature. Obtained TCS removals in the different biological treatment systems are summarized in Table 3.

Table 3 TCS removals in biological WWTPs (Thompson et al., 2005)

Wastewater Treatment Process	**TCS**			**Source**
	Influent (µg/L)	**Effluent (µg/L)**	**Removal (%)**	
Activated Sludge	21.90	1.10	95.0	Sabaliunas et al., 2003
	1.20	0.05	95.8	Bester, 2003
	5.21	0.24	95.4	McAvoy et al., 2002
	10.70	0.41	96.2	McAvoy et al., 2002
	0.67	0.03	95.2	Kanda et al., 2003
	1.10	0.03	97.5	Kanda et al., 2003
Trickling Filter	7.50	0.34	95.5	Sabaliunas et al., 2003
	3.83	1.61	58.0	McAvoy et al., 2002
	16.60	2.10	86.1	McAvoy et al., 2002
	15.40	2.70	82.5	McAvoy et al., 2002
	2.50	0.14	94.4	Kanda et al., 2003

One of the removal mechanisms of TCS in the activated sludge systems was reported to be the adsorption to the sludge to the certain extent (Singer et al., 2002; Bester, 2003; Heidler and Halden, 2007). In one other recent study,

TCS and Triclocarban were observed as the emerging trace organic compounds that were mostly sorbed to the all activated sludges among 19 trace contaminants (Hyland et al., 2012). In contrast, Federle et al. (2002) demonstrated that only 1.5-4.5% of TCS was adsorbed to the sludge and 94% of it was biodegraded in the laboratory-scale activated sludge system.

Due to the wide range of the reported adsorption percentages in different studies, the adsorption of TCS to the sludge phase during biological treatment should be carefully investigated. There are also studies in the literature demonstrating the adsorption of TCS to the laboratory materials and equipments (i.e. plastic containers and pipings, membrane and so on) and thus diverging from the exact treatment efficiencies of the applied processes (i.e. ozone treatment, NF/RO systems) when these materials and equipments were preferred in the studies focused on TCS removal from the water environment (Koc et al., 2013).

Thompson et al. (2005) further stated that TCS removal was thoroughly dependent upon the treatment process applied in biological WWTP and the retention time in the plants. This situation was also clearly seen from the data represented in Table 3. TCS removal over 95% (Bester, 2003; Kanda et al., 2003; McAvoy et al., 2002; Sabaliunas et al., 2003) was achived in the activated sludge systems including long retention times whereas lower and variable treatment efficiencies (Kanda et al., 2003; McAvoy et al., 2002; Sabaliunas et al., 2003) were observed in the systems designed to operate in shorter retention times, namely trickling filters.

On the other hand, Stasinakis et al. (2008) demonstrated that toxicity of TCS increased at higher sludge ages and the higher inhibitory effect of TCS was observed in the activated sludge systems under these circumstances, indicating that microorganisms in the activated sludge could not sufficiently remove TCS at higher sludge ages operated in the treatment systems.

In other respects, none of the studies indicated in Table 3 have considered the adaptation of microbial culture to TCS; which in fact may affect the biological TCS removal itself. However, there are some studies indicating that the adaptation of microbial cultures to the toxic contaminants due to continuous input and following acclimation period may result in the reduction or complete elimination of the compound inhibition effect (Yetiş et al., 1999; Dilek et al., 1998; Silvestre et al., 2011; Vaiopoulou and Gikas, 2012). This is also supported by another study in the literature conducted by Stasinakis et al. (2007) and they demonstrated that TCS concentration of 0.5 mg/L had adverse effects on the ammonia removal and nitrification capacity of the activated sludge system operated with non-acclimated cultures whereas these effects were not observed in the system even in the contaminant concentration of 2 mg/L following the biomass acclimation.

Moreover, certain degradation products were detected in some of the studies; however, they were not designated namely (Roy F. Weston Inc., 1992; Federle et al., 2002).

2.6 Molecular Biological Techniques

In recent years, molecular fingerprint techniques have drawn the attentions of the environmental scientists making a research on the area of biotechnology and genetic structure of the community. Thanks to these techniques, it becomes possible to reveal the short term and long term effects of serious environmental contaminants on the microorganisms and also to investigate the reactions of microorganisms to these contaminants. PCR and DGGE are one of these molecular biological techniques to identify the microbial community structure and have knowledge about the dominant microbial organisms in the system of concern. PCR and DGGE techniques are based on the principle of PCR amplification of the microorganism 16SrRNA gene and subsequent analysis by DGGE. The speciality of 16SrRNA gene is due to its stability and widely presence in most

microorganisms. Furthermore, it carries species-specific signature sequences, and thus it is commonly used to distinguish the microorganisms from each other (Canard et al., 1992).

Literature is full of studies performed with PCR and DGGE for different purposes. In one of the studies, it was found that exposure to low level of TCS by mices in their drinking food caused alterations in the intestinal bacterial populations (Pasch et al., 2009). PCR-DGGE method was also applied in another study for the determination of cyanobacteria community composition in eutrophic freshwater bodies and the major eutrophication indicating cyanobacterias were assigned in the existing conditions (Luo et al., 2014). The other study conducted by Wu et al. (2006) focuses on the designation of microbial composition of the drinking water in China by means of PCR-DGGE technique.

2.7 Microbial Kinetics

Biokinetic parameters are one of the important factors affecting the actual performance and treatment efficiency of the WWTPs. Identification of the parameters such as substrate utilization rate and bacterial growth rate has a significant role for the well understanding of fate of organic matters in the treatment systems during both the design and operation stages of the WWTPs (Şahinkaya, 2006). Thanks to these parameters, it becomes possible to optimize the biological treatment systems and then to increase the removal efficiency of the plant (Grady et al., 1996). Monod Model (Equation 1) is commonly used for the determination of the aforesaid biokinetic parameters (Ivanov, 2010).

$$\mu = \frac{\mu_{max}\, S}{K_S + S} \qquad (1)$$

Where;

μ: specific growth rate ($time^{-1}$)

μ_{max}: maximum specific growth rate ($time^{-1}$)

S: substrate concentration (mass/unit volume)

K_s: half saturation rate constant (mass/unit volume)

Influent wastewaters of WWTPs carry several carbon sources and toxic substances inside and therefore, inhibition of bacterial growth occurs especially at greater substrate concentrations indicating that Monod Model becomes inadequate for the determination of biokinetic constants (Genç, 2010). In this stage, competitive inhibition (Equation 2), non-competitive inhibition (Equation 3) or substrate inhibition (Equation 4) comes to the forefront depending upon the prevailing conditions and following the identification of the inhibition type in the light of the measurements of the microorganisms in the system, biokinetic parameters are calculated with the help of the corresponding equations derivated from the Monod Model (Ivanov, 2010).

$$\mu = \frac{\mu_{max}\, S}{\alpha K_s + S} \qquad (2)$$

$$\mu = \frac{\mu_{max}\, S}{\alpha(K_s + S)} \qquad (3)$$

$$\mu = \frac{\mu_{max}\, S}{K_s + S + (S^2/K_I)} \qquad (4)$$

Where;

α: competitive inhibition constant (unitless)

K_I: inhibition concentration of the competitive inhibitor (mass/unit volume)

The relationship between competitive inhibition constant and inhibition concentration of the competitive inhibitor is given in Equation 5.

$$\alpha = 1 + \frac{I}{K_I} \qquad (5)$$

Where;

I: concentration of the inhibitor (mass/unit volume)

Yield (Y) is another parameter used for the kinetic expressions and it is known as the biomass produced for substate utilizated by the microorganism in the biological system. Formula used for the calculation of the yield coefficient is given in Equation 6 (Ivanov, 2010).

$$Y = \frac{X_t - X_0}{S_0 - S_t} \qquad (6)$$

Where;

X_t: biomass concentration at time t (mass/unit volume)

X_0: initial biomass concentration (mass/unit volume)

S_t: concentration of substrate at time t (mass/unit volume)

S_0: initial concentration of substrate (mass/unit volume)

Specific growth rate and yield coefficient have a relationship and this relationship is explaned by the formula given in Equation 7.

$$\mu = qY \qquad (7)$$

Where;

q: specific substrate utilization rate ($time^{-1}$)

CHAPTER 3

MATERIALS AND METHODS

3.1 Source of Microbial Culture

Microbial culture used as a seed during the experiments was obtained from METU Technocity Bio-Membrane type WWTP (Vacuum Rotating Membrane). The plant is designed to serve for nearly 2000 people inhabiting in the university campus and the source of wastewater coming to the plant is of completely domestic origin. Wastewater treatment is achieved by means of a vacuum rotating membrane consisting of ultrafiltration membranes submerged within the activated sludge unit. The logic under this way of treatment is based upon biological treatment as in the case of conventional activated sludge system; however, it is superior due to capability of bacteria and virus removal up to 99.9% and transparency of effluent water achieved at the end. After the treatment, effluent water of the plant is used for the irrigation of lawns in the campus area.

During the sampling, sludge samples were taken from the effluent of membrane bioreactor and put into dark colored glass bottles having a volume of 2 L. Immediately after, samples were delivered to the laboratory for the experiments.

3.2 Synthetic Wastewater

Composition of the synthetic wastewater used is provided in Table 4. TCS was spiked into this wastewater to obtain different TCS levels. Spiking with TCS was accomplished using TCS stock solution which was prepared in

ultra-pure water containing 0.04 M sodium hydroxide using 100 mL volumetric flasks made of borosilicate glass (Pyrex) and stored in refrigerator at +4°C in dark.

Table 4 Composition of synthetic wastewater

Ingredient	Concentration (mg/L)
Proteous-peptone	470
NaCl	156.70
Na_2SO_4	17.20
K_2HPO_4	44.60
$MgCl_2.6H_2O$	3.700
$FeCl_2.4H_2O$	4.520
$CaCl_2$	2.794
$MnSO_4.H_2O$	0.0638
$ZnSO_4.7H_2O$	0.0819
$CoCl_2.6H_2O$	0.0753
$CuSO_4$	0.0760
$(NH_4)_6Mo_7O_{24}.4H_20$	0.0338

3.3 Chemicals

Chemicals used during the experiments were namely HPLC grade methanol (Merck, >99.9% purity), HPLC grade acetonitrile (Merck), sodium hydroxide (Sigma-Aldrich, ≥97% purity), proteous-peptone (Oxoid) and synthetic wastewater minerals (as indicated in Table 4), standards of TCS (Irgasan; Sigma-Aldrich, ≥97% purity), 2,4-dichlorophenol (Sigma-Aldrich, 99% purity), 4-chlorocathecol (Sigma-Aldrich, 97% purity), 2, 4-dichloroanisole (Sigma Aldrich, 97% purity) and 4-chlororesorcinol (Sigma Aldrich, 98% purity).

3.4 Laboratory Devices, Equipments and Labwares

Laboratory devices and equipments utilized during the experiments are listed in Table 5 with the intended use of each.

Table 5 Laboratory devices and equipments used in the study

Name/Model	**Intended Use**
HPLC- SHIMADZU, LC 10AT	TCS analysis
Ultra-pure Water Device/Human Power I+ Scholer ve Millipore Simplicity 188	Obtainment of ultra-pure water required for almost all experiments and analyses
Muffle Furnace/Lenton	MLVSS analysis of the samples taken from the reactor
Magnetic Stirrer/WiseStir, MSH-20A	Preparation of solution and some analytes
Analytical Balance/GEC AVERY	Preparation of solution, measurement of MLSS and MLVSS
Centrifuge/Hettic rotofix 32A	Separation of solid phase from the liquid phase for the samples taken from the reactors
Refrigerator	Safely storage of the nutrient solutions to be used in the reactors, conservation of sludge and DNA samples
HACH Reactor	COD analysis
HACH DR/2500 Spectrophotometer	COD analysis
Soxhlet Extraction Device/ Gerhardt	Extraction of TCS from sludge

Table 5 Laboratory devices and equipments used in the study (Cont'd)

Name/Model	**Intended Use**
PCR Device (Istanbul Medeniyet University Bioengineering Department)	Replication of extracted DNA
DGGE Device (Istanbul Medeniyet University Bioengineering Department)	Denaturation of DNA replicated by PCR
Gel Electrophoresis Device (Istanbul Medeniyet University Bioengineering Department)	Quality control of the DNA replicated by PCR
Gel Screening Device (Istanbul Medeniyet University Bioengineering Department)	Screening of the gel electrophoresis and DGGE bands

All labwares (glass pipettes, dark glass bottles, beakers, etc.) used in the studies were cleaned prior to experiments to eliminate any associated pollution interference (i.e. impurities etc) and ultimately to obtain safe and reliable data sets. Cleansing was carried out by means of washing the labwares with a detergent called Alconox, rinsing with hot tap water and ultra-pure water, and final rinsing with HPLC grade methanol, respectively. After the cleanup procedure, the labwares were dried at 105°C for 1 h before the usage.

3.5 Analytical Methods

Measurements of MLSS and MLVSS concentrations were conducted in accordance with Standards Methods of 2450 B and 2450 E (APHA, 1998). In this context, wastewater sample (10 mL) taken from the reactor was passed through 0.45 μm pore sized filter by means of vacuum, filtration process was sustained until no water was left on filter, and filter was dried at 105°C oven for 1 h. Afterwards, it was kept in desiccator for 30 minutes in

order to be cooled down and not to be affected from the humidity, and subsequently MLSS measurement was done by weighing it on the analytical balance. For the MLVSS measurement, this filter was ignited at 600°C for 30 minutes, cooled down in the desiccators and analysis was conducted ultimately.

COD measurements were achieved with the help of Hach Lange vials based upon EPA approved HACH 8000 Method (HACH Water Analysis Handbook, 1992). Within this scope, 2 mL wastewater sample was put into vial after passed through the vacuum filter, it was digested for 2 h in HACH reactor, and COD concentration of the sample was then determined by a HACH DR/2500 spectrophotometer.

TCS concentration was measured using a high performance liquid chromatography device (HPLC- SHIMADZU, LC 10AT, Tokyo, Japan) which is equipped with Nucleosil C18 column (4.6 mm × 250 mm), LC-10Atvp solvent delivery module, an SCL-10Avp system controller and a SPD-10Avp UV-VIS detector set at 280 nm (Figure 2). For the analysis of TCS with this device, there was a need to find or develop a suitable method. As a first, detailed literature survey was conducted, and lots of methods developed earlier were tried by using methanol, ultra-pure water and acetonitrile as solvents with different fractions. Since, no success was achieved with the implementation of existing methods, some modifications were tried on them with and without buffer solution application, and at the end, the method involving acetonitrile (75%) and ultra-pure water (25%) as solvents at a constant flow rate of 1.5 mL/min was found to be the well-suited method for TCS measurements. Injection volume of sample was set as 20 µL. Retention time of TCS was obtained as 5.3-5.6 min. Possible by-product formation was also monitored by the HPLC-UV device.

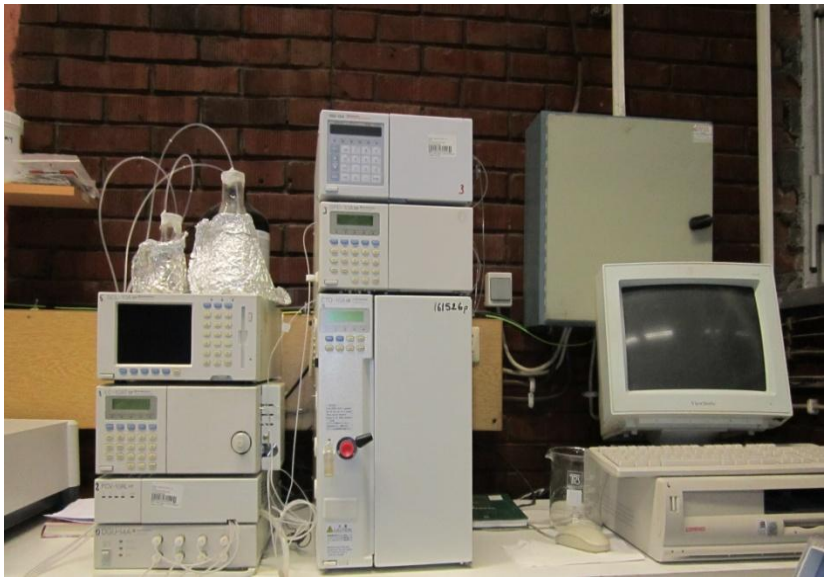


Figure 2 HPLC-UV device

3.6 Experiments

3.6.1 Pulse-fed Sequencing Batch Reactor Operations

Pulse-fed sequencing batch reactor operations were carried out with the sludge samples taken from the METU Technocity Membrane Treatment Plant. A laboratory scale biological reactor was operated with the aforesaid sludge sample and it was fed by the synthetic wastewater bearing TCS at different concentrations (100 ng/L-100 mg/L) on a daily basis.

TCS concentration range studied in this set of experiments was determined with the consideration of both TCS toxicity threshold values (i.e. EC_{50}, LC_{50}) reported in the literature for various organisms and TCS concentrations likely to be encountered in wastewaters. At this stage, the TCS concentration range of 100 ng/L-100 mg/L was designated as the working range to be run during the experiments. However, since the limit of detection (LOD) value is 10 µg/L for the method applied in the measurement of TCS with the HPLC-UV device and the lower value of the designated working range (100 ng/L) falls below this concentration, it might not be possible to get sensitive and measurable results after biological

treatment for the influent contaminant concentrations of 100 ng/L, 100 μg/L and 500 μg/L among the designated range. It was also confirmed with the experiments conducted with these influent TCS concentrations (100 ng/L, 100 μg/L and 500 μg/L) and the results showed that effluent water had TCS contents lower than detection value at steady state conditions. In this respect, aforementioned TCS concentrations (100 ng/L, 100 μg/L and 500 μg/L) were applied for the monitoring of the acclimation of microbial culture to the contaminant and for the investigation of the effects on the general performance of the activated sludge. In other words, removal efficiency, fate and kinetics of TCS in the system were not taken into consideration for these influent concentrations. When the steady state condition was satisfied in the reactor, COD, MLSS, pH and sludge volume index (SVI) were monitored for the influent TCS concentrations less than 1 mg/L while TCS concentration (both in wastewater and sludge) was measured additionally for the ones equal to and greater than 1 mg/L.

Proteous-peptone was used for the carbon and energy source during the experiments and it was added to the synthetic wastewater until 500 mg/L COD content was attained in the wastewater. Dark coloured glass bottles with a volume of 2.5 L (2 L net volume) were used as reactors (Figure 3). Dark coloured glass bottles, rather than plastic bottles, were preferred in order to avoid the adsorption of highly hydrophobic natured TCS to the plastic materials and equipments, and to prevent potential photodegradation. Liquid volume in the reactor was arranged through the addition of 1 L sludge sample and 1 L synthetic wastewater.

In order to operate numerous reactors simultaneously and shorten the research time as far as possible, pulse-fed sequencing batch reactor was utilized instead of continuous system considering the pump requirement and so on. Reactors, laboratory scale activated sludge units, were operated at a sludge age of 8 d (SRT) in fill and draw mode to simulate a real WWTP. So as to provide aforesaid sludge age, 250 mL of the wastewater is wasted from

the reactor, the reactor is allowed to settle for 30 minutes, 750 mL of the clear effluent is drained out of the reactor and 1 L synthetic wastewater is added to the reactor to keep the volume constant at 2 L, respectively on a daily basis. Reactors were placed into the water-baths, run in the temperature of 25°C and air was provided by means of the air pumps.

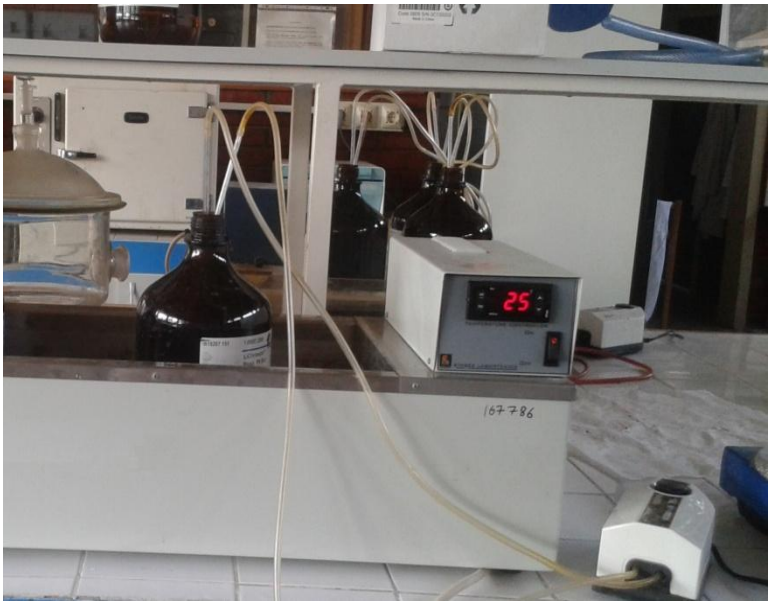


Figure 3 Pulse-fed sequencing batch reactor operated with acclimated culture

When the reactor was observed to reach the steady state condition through the daily COD and MLSS measurements, analysis of TCS, COD, MLSS, pH and SVI was done for the samples taken from the outlet of the reactor. TCS measurements of wastewater and sludge samples taken from the reactor were accomplished by HPLC-UV device and mass balance of TCS was formed in order to comprehend the removal mechanism of TCS in the system. Prior to TCS measurement, wastewater samples were passed through 0.45 µm pore sized filters and subsequently analyzed for TCS. On the other hand, sludge samples were extracted via Soxhlet apparatus (Figure 4) using methanol as an extraction solvent and righ after passed through the 0.45 µm pore sized filter before TCS analysis. Possible by-product formation was also monitored thanks to these measurements. Furthermore,

studies were performed on the sludge samples taken from the reactor at steady state condition for the identification of microbial population composition by molecular biological techniques.

Figure 4 Soxhlet aparatus

Aforementioned pulse-fed sequencing batch reactor operations were performed with the microbial cultures that were both acclimated and non-acclimated to TCS. The acclimation of microbial culture to TCS has been provided through intermittently and gradually increased influent TCS concentrations during the experiments conducted with the acclimated cultures. In this sense, the reactor was primarily operated without TCS and when the steady state condition was observed in this condition, the reactor was started to be daily fed by the synthetic wastewater spiked with 100 ng/L TCS. Similarly, following the steady state condition in the presence of 100 ng/L influent TCS concentration within the reactor, TCS content of the synthetic wastewater was increased gradually and the reactor was fed by TCS bearing synthetic wastewater at concentrations of 100 µg/L, 500 µg/L, 1 mg/L, 10 mg/L, 20 mg/L, 50 mg/L and 100 mg/L, respectively. 100 ng/L, 100 µg/L and 500 µg/L TCS concentrations were applied for the assurance of the microbial culture acclimation to the contaminant and mass balance of

TCS was not constituted for such contaminant initial concentrations. The reactor was operated for the acclimated culture during 6 months in this way.

On the other hand, 5 separate reactors were operated in the similar conditions concurrently for the experiments conducted with the non-acclimated cultures during 1 month. Each reactor was instantly fed by the synthetic wastewater bearing different TCS concentrations (1, 10, 20, 50 and 100 mg/L) as a shock load. Since the acclimation was not a concern for these cultures, TCS concentration range of 100 ng/L-500 µg/L was not studied in this set of experiments.

During the experiments conducted with the non-acclimated cultures, a technical failure was observed in the HPLC-UV device and the problem was solved with replacing the one component of the detector with the new one. In this stage, the software of the device was amended and the detection time of TCS with the existing method was shifted to 5.5-5.8 min accordingly.

3.6.1.1 Determination of Microbial Composition

Sludge samples taken from the pulse-fed sequencing batch reactors operated with raw sludge (free of TCS), acclimated (100 ng/L-100 mg/L TCS) and non-acclimated cultures (1-100 mg/L TCS) following the steady state conditions were conveyed to the Bioengineering Department of Istanbul Medeniyet University where microbial composition determination studies were kindly performed using molecular biological techniques. During these studies, firstly, DNA extraction was performed for these sludge samples. After extraction, 16SrDNA samples that were isolated from the cultures using universal primers were amplified by combined PCR-culture technique. In the second stage, alteration of the bacterial composition incidental to biofilm was determined by means of DGGE. For the sequencing of the purified products, service was bought from REFGEN (Ankara, Turkey), the company placed in METU Technocity and serving for

biotechnological researches. After sequence analysis, determination of microbial composition was carried out by BLAST (Basic Local Alignment Search Tool) program, nucleotide/protein sequence database that is free and online service from National Center for Biotechnology Information.

Detailed information regarding the materials and methods used in these molecular biological techniques (i.e. PCR and DGGE) are presented in Appendix A.

3.6.2 Kinetics Studies

Batch reactors were operated to study the kinetics of TCS removal. To this end, Erlenmeyer flasks having a volume of 500 mL were used and the liquid volume in the flasks was set to 250 mL by means of tap water. The openings of the flasks were coated with cotton and experiments were conducted on an orbital shaking incubator at 25°C and 250 rpm of mixing speed (Figure 5). The experiments were made both for acclimated and non-acclimated microbial culture.

Sludge samples previously taken from the pulse-fed sequencing batch reactors for each influent TCS concentration after steady state condition were two times washed with ultra-pure water and thereby the organic matters that are sorbed to the surface of microorganisms were removed safely. The gathered cultures were then mixed with a slight amount of ultra-pure water and COD/biomass ratio was fixed to be lower than 0.4 for each flask.

The experiments were accomplished with the synthetic wastewater prepared both in the presence and absence of proteous-peptone so that two scenarios as TCS being a sole carbon and energy source in the water environment and as in addition to TCS presence of the proteous-peptone in the water environment and the effect of TCS on the utilization of this easily

biodegradable carbon and energy source were studied separately. Meanwhile, the deficiency of the nitrogen owing to proteous-peptone exclusion from the synthetic wastewater was met with the addition of NH_4Cl.

In total, 21 batch reactors were operated by this way: TCS-free control reactor, 5 reactors in the presence of both TCS and proteous-peptone and including the microbial cultures that were already acclimated to TCS at different concentrations (1, 10, 20, 50 and 100 mg/L), 5 reactors in the presence of TCS as a sole carbon and energy source and including the microbial cultures that were acclimated to TCS at different concentrations (1, 10, 20, 50 and 100 mg/L), 5 reactors in the presence of both TCS and proteous-peptone and including the microbial cultures that were shock-loaded to TCS at different concentrations (1, 10, 20, 50 and 100 mg/L) and 5 reactors in the presence of TCS as a sole carbon and energy source and including the microbial cultures that were shock-loaded to TCS at different concentrations (1, 10, 20, 50 and 100 mg/L), respectively.

At certain time intervals, samples were taken from the flasks and they were analyzed for COD, MLSS and TCS. Finally, the obtained data was used for the determination of biokinetic constants (q_{max}, μ_{max}, Y, K_s and K_I) by using the Equations 1-7.

Figure 5 Batch experimental set-up

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Pulse-fed Sequencing Batch Reactor Operations

4.1.1 Acclimated Culture

Steady state COD, MLSS, SVI and pH values are represented in Table 6 for the reactor operated with the acclimated culture in the presence of 100 ng/L-100 mg/L TCS concentration range and run in a fill and draw mode during 6 months.

Variations in COD and MLSS concentrations during the steady state conditions in the system are demonstrated on Figure 6 for different influent TCS concentrations.

Table 6 Steady state characteristics of the reactor operated with TCS-acclimated culture (100 ng/L-100 mg/L influent TCS concentrations)

TCS	COD (mg/L)	MLSS (mg/L)	SVI (mL/g)	pH
0	30	1000	54.17	7.40
100 ng/L	35	1020	53.10	7.39
100 µg/L	37	1080	54.01	7.39
500 µg/L	45	1280	52.08	7.40
1 mg/L	78	280	59.52	7.41
10 mg/L	94	170	122.55	7.48
20 mg/L	162	380	76.75	7.50
50 mg/L	211	200	93.75	7.51
100 mg/L	263	130	96.15	7.56

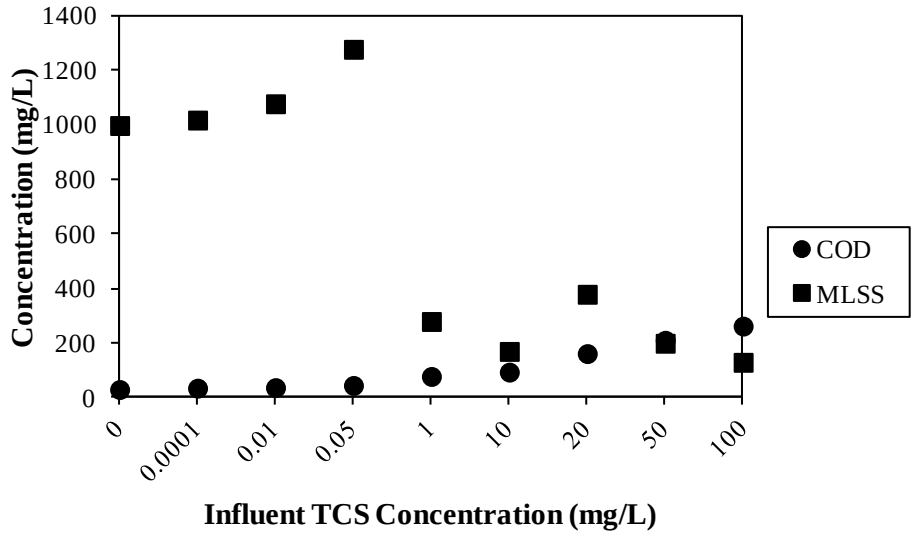


Figure 6 COD and MLSS concentration variations with influent TCS concentration in the pulse-fed sequencing batch reactor (Acclimated culture)

In order to see the effect of TCS on the COD removal performance of the system, COD removal efficiency with the corresponding influent TCS concentration in the reactor is drawn and illustrated in Table 7.

Table 7 COD removal efficiency in the pulse-fed sequencing batch reactor (Acclimated culture)

Influent TCS Concentration	**COD Removal (%)**
0	94.0
100 ng/L	93.0
100 µg/L	92.6
500 µg/L	91.0
1 mg/L	84.4
10 mg/L	81.2
20 mg/L	67.6
50 mg/L	57.8
100 mg/L	47.4

As can be seen from Table 6 and Figure 6, observed COD values at steady state conditions were increasing with the increased TCS input to the reactor. In other words, COD removal performance of the system was reduced with the increased TCS concentration. In the case that no TCS was supplied to the reactor, effluent COD concentration was found as 30 mg/L; however, it gradually increased with the abundancy of TCS in the system. Although this increase was not so apparent up to 1 mg/L influent TCS concentration, there observed dramatic increase in the effluent COD concentration with the greater contaminant dosages. Similarly, it was clearly inferred from Table 7 that COD removal efficiency of the system reduced to almost half value (47.4%) at an influent TCS concentration of 100 mg/L compared to the COD removal (94%) observed in the absence of TCS in the reactor. This situation can be interpreted as that TCS in greater concentrations was toxic to microbial culture and thus, the microbial community became sensitive to the environmental conditions and stresses they faced with and immediately after incapable of substrate utilization (proteous-peptone) which obliquely affecting the COD removal efficiency of the system in an adverse manner. From this point of view, it was a fact to bear in mind that discharge of TCS containing wastewaters to the WWTPs lead to the significant decreases in the actual performance of the plants and ultimately result in undesired effluent concentrations that are not complied with the existing discharge standards by impairing the water quality of the receiving waters.

In order to comprehend the fate of the contaminant in the liquid and solid phases, mass balance was constructed by means of TCS analysis of the effluent water and sludge samples for the influent TCS concentrations equal to and greater than 1 mg/L when the steady state condition was reached in the reactor. Mass balance was formed with respect to Equation 8.

$$M_{in} = M_{out} + M_{sludge} + M_{removed} \qquad (8)$$

Where;

M_{in}: TCS mass supplied to the activated sludge reactor until steady state (mg)

M_{out}: Total TCS mass remaining in the liquid phase of the reactor at steady state (mg)

M_{sludge}: Total TCS mass in the biomass of the activated sludge reactor at steady state (mg)

$M_{removed}$: Mass of TCS that is biologically removed/treated in the reactor (mg)

HPLC-UV chromatograms, which were demonstrating TCS analysis results of the effluent water and sludge samples taken from the reactor following the steady state conditions for each influent TCS concentration (i.e. ≥ 1 mg/L), are provided in Section 4.1.3. TCS retention time was observed in the range of 5.3-5.6 min on these chromotagrams.

Mass balance analysis that was constituted based upon both the formulation given in Equation 8 and the chromatograms provided for the effluent water and sludge samples in Section 4.1.3 is summarized in Table 8.

Table 8 TCS mass balance for the reactor operated with TCS-acclimated microbial culture

M_{in}		**M_{sludge}**		**M_{out}**		**$M_{removed}$ (biodegradation) (mg)**	**TCS Biodegraded %**	**TCS Removal from Water %**
mg	**mg/L**	**mg**	**mg/L***	**mg**	**mg/L**			
2	1	0.06	0.03	0	0	1.94	97.0	100.0
20	10	1.33	0.67	0.07	0.035	18.60	93.0	99.7
40	20	2.36	1.18	5.74	2.870	31.90	79.8	85.7
100	50	6.00	3.00	19.13	9.565	74.87	74.9	80.9
200	100	47.06	23.53	28.32	14.160	124.62	62.3	85.8

* Calculated by dividing the calculated mass with a reactor volume of 2 L.

Biological TCS removal efficiency of the system decreased with the increased TCS input to the reactor, as can be demonstrated in Table 8. For instance, TCS was biologically removed with a percentage of 97% in the system when the reactor was initially fed with 1 mg/L TCS concentration whereas the biodegradation performance of the system was gradually deteriorated and the removal percentage was decreased down to 80% and almost 63% with the TCS input of 20 mg/L and 100 mg/L, respectively.

TCS concentrations of the effluent water and sludge samples taken from the reactor and the corresponding TCS biodegradation efficiencies following the steady state conditions attained for different TCS inputs are represented in Figure 7.

For the assessment of TCS removed from water environment, it was found with this study that TCS was completely treated (i.e. 100% removal efficiency) from water phase and the reactor effluent water was free of TCS for the initial TCS concentrations of 1 mg/L. However, with the increased dosages as 20 mg/L and 100 mg/L TCS, the removal percentages of the parent compound were observed around 85% and the contaminant concentration was dependently increased in the effluent water.

Lots of the studies in the literature were conducted by ignoring the hydrophobic characteristic of TCS (Kanda et al., 2003; Sabaliunas et al., 2003) and therefore, high TCS removal efficiencies were reported in the effluents of treatment plants without TCS analysis in sewage sludge. However, it makes no sense to state that such removal efficiencies were satisfied in the treatment plant effluents and it does not reflect the truth any more without considering the adsorption of TCS to sewage sludge due to its high hydrophobic nature and tendency to accumulate on solid phases.

In this respect, while the assessment of TCS removal in WWTPs, it is not adequate to monitor influent and effluent water and sewage sludge should

also be analyzed for TCS at the same time in order to achieve true understanding of the removal mechanism and to obtain accurate removal results.

Additionally, TCS can be transformed into any other toxic substances during the treatment process as is known and thus, the obtained removal percentages should be evaluated in company with its metabolites and their concentrations observed in this study at the end of Section 4.1.3.

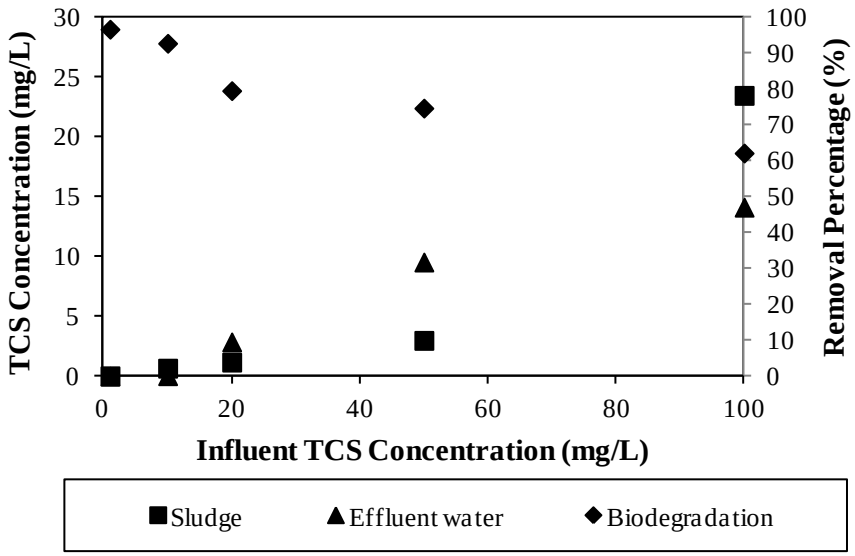


Figure 7 TCS concentrations of effluent water and sludge samples and TCS biodegradation efficiencies in the pulse-fed sequencing batch reactor at steady state (Acclimated culture)

Percentage of TCS in effluent water and sludge samples after treatment and corresponding biodegradation efficiencies based upon the TCS input to the reactor are delineated in Figure 8.

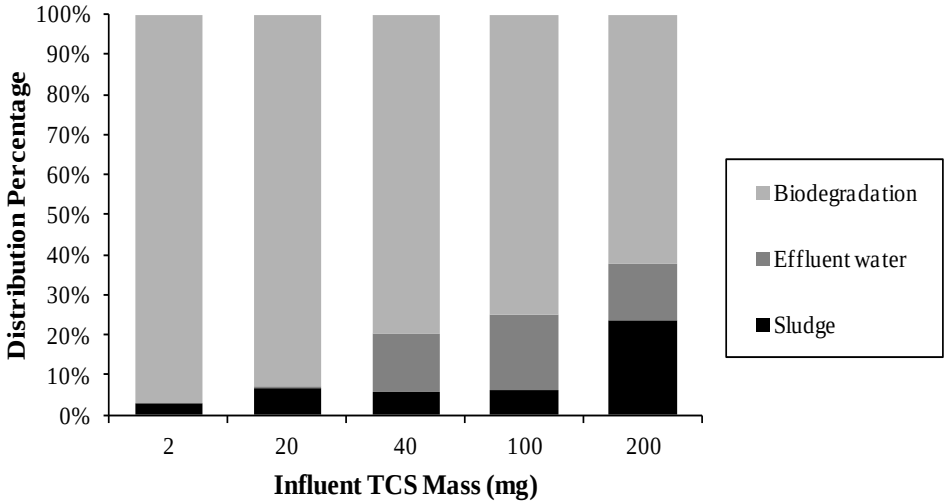


Figure 8 Distribution percentage of TCS in effluent water, sludge and as biodegraded in the fed-bath reactor (Acclimated culture)

As can be seen from Figure 8, biological removal efficiency was entirely related with the influent TCS concentration and it was decreasing when greater amount of TCS was supplied to the reactor. In the circumstances, the amount of TCS remaining in the liquid phase after aerobic treatment was increasing as expectedly. When 2 mg (i.e. 1 mg/L) TCS was spiked to the reactor, no TCS was observed in effluent water, 3% of TCS was sorbed to the sludge and 97% of it was removed by means of biodegradation in the system. On the other hand, following TCS input of 200 mg (i.e. 100 mg/L), there was a significant decrease in the removal efficiency and 14% of TCS was remained in effluent water, 24% of it was sorbed to sludge and the rest was biologically remained in the system.

4.1.2 Non-acclimated Culture

Stedy state COD, MLSS, SVI and pH values are summarized in Table 9 for the reactors operated with the non-acclimated culture in the presence of 1 mg/L-100 mg/L TCS concentration range and run in a semi batch mode during 1 month period.

Alterations in COD and MLSS concentrations following the steady state conditions in the system are represented on Figure 9 for different influent TCS concentrations.

Table 9 Steady state values of the reactors operated with TCS non-acclimated culture (1 mg/L-100 mg/L influent TCS concentrations)

TCS (mg/L)	COD (mg/L)	MLSS (mg/L)	SVI (mL/g)	pH
0	30	1000	54.17	7.40
1	170	200	104.17	7.39
10	186	150	111.11	7.40
20	208	130	96.15	7.43
50	214	120	86.81	7.47
100	289	100	83.33	7.50

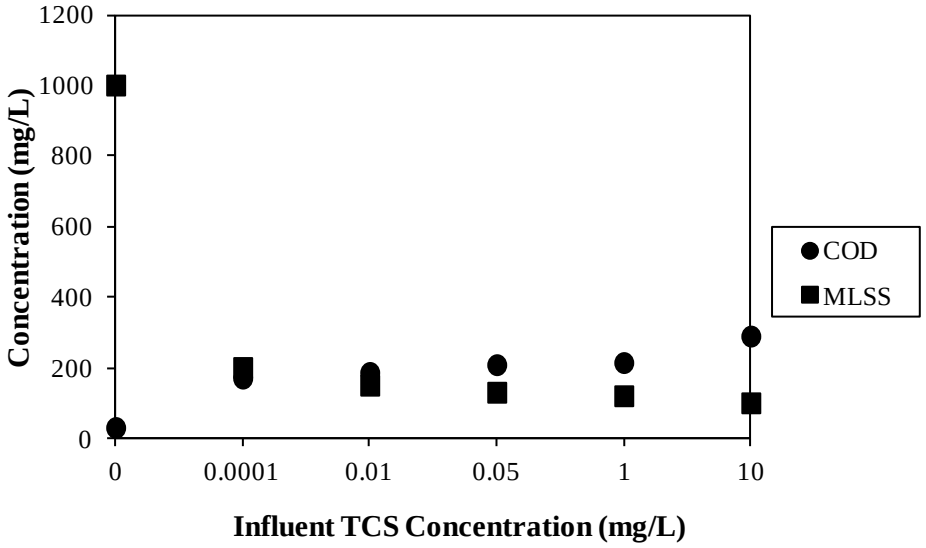


Figure 9 COD and MLSS variations in the pulse-fed sequencing batch reactors (Non-acclimated culture)

In order to see the effect of shock loading of TCS on the COD removal performance of the system, Table 10 is prepared for the illustration of COD removal efficiency corresponding to influent TCS concentration in the reactors.

Table 10 COD removal efficiency in the pulse-fed sequencing batch reactors (Non-acclimated culture)

Influent TCS Concentration (mg/L)	**COD Removal (%)**
0	94.0
1	66.0
10	62.8
20	58.4
50	57.2
100	42.2

As can be inferred from Table 9 and Figure 9, effluent COD values at steady state conditions were entirely affected with the influent TCS concentration and it was rising with the increased TCS input to the system. Similar to the experiments conducted with the acclimated culture, there observed a serious drop in COD removal efficiency of the system when the contaminant was fed in greater concentration. Activated sludge system was observed to have an actual COD removal efficiency of 94% in the absence of TCS within the system; however, this COD removal efficiency declined sharply and it was found as 66% following the shock loading of raw sludge with 1 mg/L TCS. Furthermore, when 100 mg/L TCS was suddenly introduced to the raw sludge, COD removal efficiency of the system decreased dramatically and was obtained to be lower than half of the value (42.2%) gathered in TCS-free environment (Table 10). This situation might be due to the toxicity of TCS to microbial culture when it was excessive in the environment, the sensitivity of the microbial community to the environmental stress associated with the aforesaid contaminant, the immobilization of substrate utilization (proteous-peptone) and thus the deterioration of COD removal performance of the system as expected at the end. The reflection of this situation to the daily life may be through the arrival of TCS bearing wastewaters to the WWTPs via sudden and intermittent discharges and so

the inhibition of the biological treatment units' performance which brings about a decrease in the removal efficiency of the system in final.

There was an observed trend toward decreased COD removal efficiency of the activated sludge system operated with non-acclimated microbial culture when compared to the reactor operated with acclimated culture. This decrease was apparent at the reactors operated with non-acclimated culture for all studied influent TCS concentrations. However, the effect was less pronounced for influent TCS concentration above 20 mg/L and COD removal efficiency of the system was less affected from the acclimation for higher influent TCS concentrations. To illustrate, 84% of COD was utilized and removed in the reactor including microbial culture acclimated to 1 mg/L TCS whereas this ratio was observed as 66% in the case of shock loading with the same amount of contaminant. On the other hand, COD removal efficiencies of 47.4% and 42.2% were beholded for the reactors operated with acclimated and non-acclimated culture in the presence of 100 mg/L TCS, respectively. This situation could be explained with the diminishment in the impact of acclimation on COD removal efficiency of the biological treatment system above certain contaminant dosages. Nevertheless, since more COD reduction was achieved with prior TCS acclimation, the statements in the literature regarding the positive effects of contaminant acclimation on the general treatment performance of the biological system in terms of conventional parameters (Yetiş et al., 1999; Dilek et al., 1998; Şahinkaya and Dilek, 2006 and 2007; Silvestre et al., 2011; Stasinakis et al., 2007; Vaiopoulou and Gikas, 2012) were confirmed with the foundings in this study. In other words, after certain acclimation period, the presence of contaminant might not pose a risk for the treatment system and sometimes might be affirmative for the routine and healthy operation of the system and so it may be of an option in some circumstances rather than pulse discharges from the industries.

HPLC-UV chromatograms, which were indicating the outcomes of TCS analysis on the effluent water and sludge samples taken from the reactor following the steady state conditions for each influent TCS concentration, are represented in Section 4.1.3. TCS retention time was observed in the range of 5.5-5.8 min on these chromotagrams.

TCS mass balance analysis that was formed according to both the formulation given in Equation 8 and the chromatograms presented for the effluent water and sludge samples in Section 4.1.3 is summarized in Table 11.

Table 11 TCS mass balance for the reactors operated with TCS non-acclimated microbial culture

M_{in}		**M_{sludge}**		**M_{out}**		**$M_{removed}$ (biodegradation) (mg)**	**TCS Biodegraded %**	**TCS Removal from Water %**
mg	**mg/L**	**mg**	**mg/L***	**mg**	**mg/L**			
2	1	0.072	0.036	0.04	0.02	1.89	94.40	98.00
20	10	0.264	0.132	1.18	0.59	18.56	92.78	94.10
40	20	10.360	5.180	11.12	5.56	18.52	46.30	72.20
100	50	42.060	21.030	14.70	7.35	43.24	43.24	85.30
200	100	94.520	47.260	21.05	10.53	84.43	42.22	89.48

* Calculated by dividing the calculated mass with a reactor volume of 2 L.

As can be seen from Table 11, biological TCS removal efficiency of the system was considerably decreasing with the increased TCS input in the shock loads to the reactor. For instance, TCS was biologically removed with a percentage of around 94% in the system while sudden introduction of 1 mg/L TCS to the reactor and this biodegradation efficiency slightly decreased and obtained as almost 93% with the pulse dosage of 10 mg/L TCS to the reactor. However, the biodegradation performance of the system was rapidly deteriorated and dramatic decreases were observed in the

removal percentages following the shock loading of the reactors with 20 mg/L, 50 mg/L and 100 mg/L TCS concentrations. At this stage, the removal percentage of 93% that was gathered in the presence of 10 mg/L TCS decreased to half value and obtained around 46% with the sudden introduction of 20 mg/L TCS. With the further increase of contaminant dosage up to 100 mg/L, TCS biodegradation efficiency was reduced down to 42%. From these foundings, it can be concluded that TCS had an acute toxicity on certain microbial community in the biological system in the concentration range of 10-20 mg/L when no acclimation period was of consideration and therefore, it was not easily utilized and removed by these types of microorganisms and the biological removal efficiency of the system was decreased accordingly.

TCS concentrations of the effluent water and sludge samples taken from the reactor and the corresponding TCS biodegradation efficiencies following the steady state conditions attained for different TCS inputs are represented in Figure 10. However, these observed removal efficiencies should be considered and interpreted in company with the metabolites of TCS and their corresponding concentrations provided in this study at the end of Section 4.1.3.

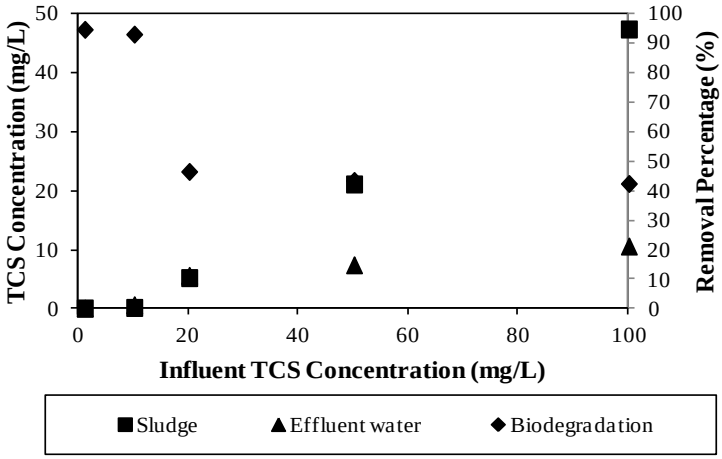


Figure 10 TCS concentrations of effluent water and sludge samples and TCS biodegradation efficiencies in the pulse-fed sequencing batch reactors at steady state (Non-acclimated culture)

Percentage of TCS in effluent water and sludge samples after treatment and corresponding biodegradation efficiencies based upon the TCS input to the reactor are delineated in Figure 11.

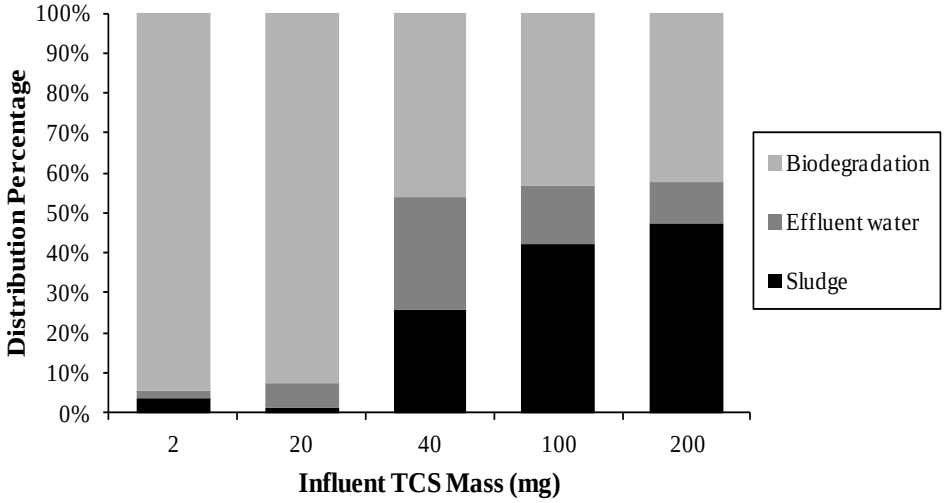


Figure 11 Distribution percentage of TCS in effluent water, sludge and as biodegraded in the pulse-fed sequencing batch reactors (Non-acclimated culture)

As illustrated in Figure 11, biological removal efficiency was decreasing in a considerable extent when the greater amount of TCS was fed to the reactor instantly. By the way, the portion of TCS that was sorbed to the sludge was increased. When 2 mg (i.e. 1 mg/L) TCS was suddenly spiked to the reactor, 3% of TCS was observed in effluent water, 3.6% of it was remained as sorbed to the sludge and 94.4% of it was biologically removed in the system. On the other side, following TCS input of 200 mg (i.e. 100 mg/L), there was a dramatic decrease in the biological removal efficiency and 11% of TCS was remained in effluent water, 47% of it was sorbed to sludge and the rest was biodegraded in the system.

Along with the biological TCS removal, whether this way of treatment brings about any other by-product and what about their fates in the pre-existing system are answered in detail in Section 4.1.3.

When the observed results were compared with the set of experiments conducted with acclimated culture, lower biological TCS removals were achieved with the reactors including non-acclimated culture. This situation was observed for all TCS concentrations worked in this study. Moreover, sharp decreases in TCS biodegradation percentage were seen especially for the non-acclimated culture experiments performed with instant 1 mg/L and 100 mg/L TCS concentrations, as 94.40% and 42.22%, respectively. On the other hand, TCS biodegration performance of the system was less affected for the experiments conducted with acclimated culture with the same contaminant concentrations (i.e. 97.0% and 62.3%).

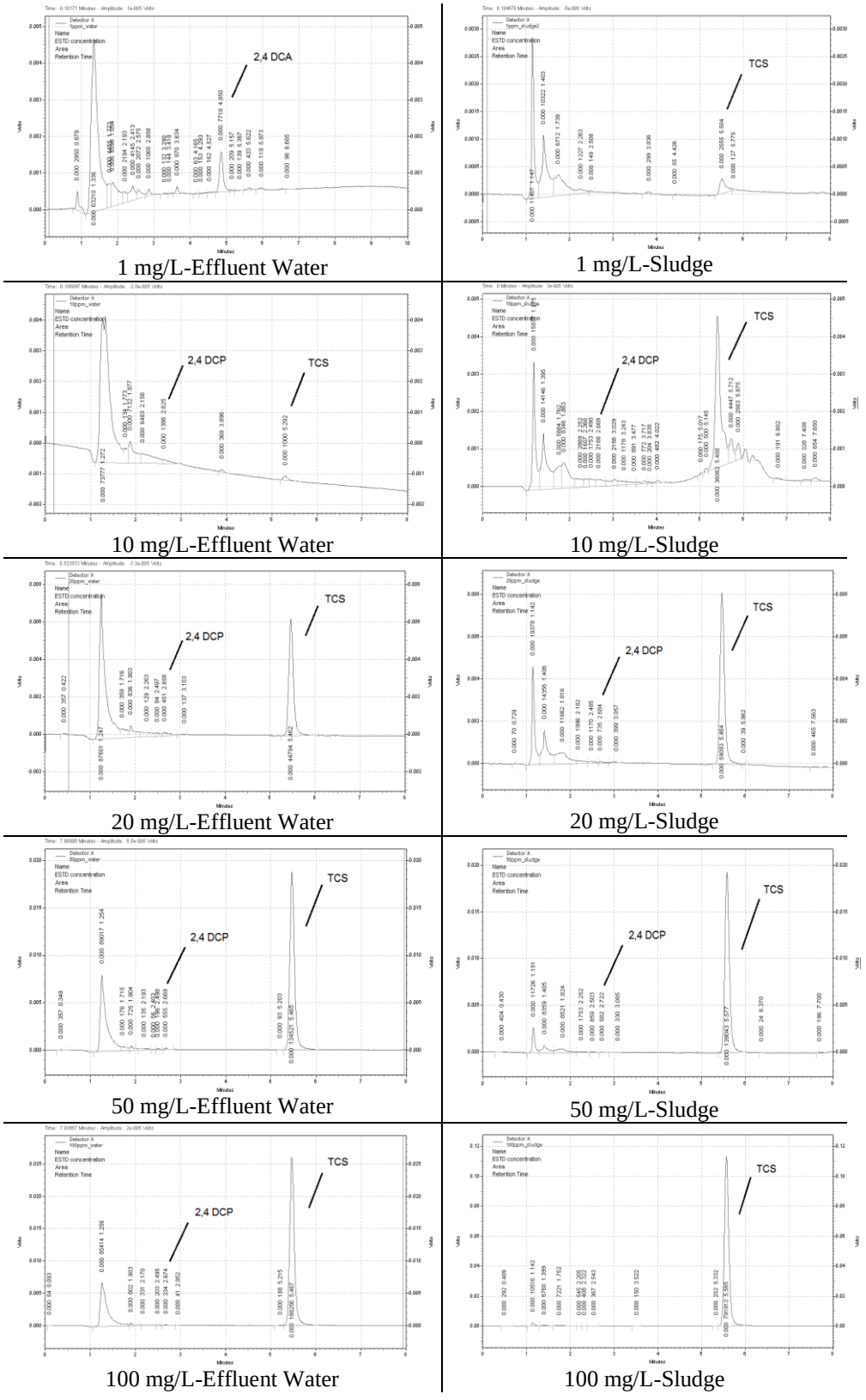
4.1.3 By-product Analysis

It is known that TCS may transform into different by-products as a result of biological degradation process. Concerning the subject, there were studies in the literature indicating the conversion of TCS to more toxic substances like chlorinated phenols and trihalomethanes after the chlorination process applied especially in WWTPs and the transformation into the subtances such as dioxins, 2,4-dichlorophenol and 2,4,6-trichlorophenol in the environment owing to different processes (Kanetoshi et al, 1987; Rule et al, 2005).

In the light of these findings in the literature, the determination of the potential metabolites arising due to biological degradation of TCS within the system have become an important issue and further investigations were conducted with the acclimated and non-acclimated microbial cultures for an ultimate aim of designating by-products both as identity and quantity. Within this scope, HPLC-UV chromatograms (i.e. Figure 12 and Figure 13) belonging to TCS analyses on effluent water and sludge samples that were taken from the pulse-fed sequencing batch reactors operated for acclimated and non-acclimated cultures following the steady state condition were

examined and all of the peaks observed in these chromatograms were identified with their relevant retention times.

As can be inferred from the chromatograms represented in Figure 12 and Figure 13, there were other different peaks than TCS on the effluent water and sludge samples taken from the reactors at steady state. For the determination of the substances corresponding to these peaks, detailed literature survey was conducted as an initial step and then the standards of 2,4-dichlorophenol (2,4-DCP), 4-chlorocatechol (4-CC), 4-chlororesorcinol (4-CR) and 2,4-dichloroanisole (2,4-DCA) were purchased to be used in the control experiments. Afterward, these standards were given to the HPLC-UV device, their chromatograms were obtained and the arrival times of their peaks were detected. In this way, arrival times of each standard were compared with the retention times of the peaks observed in the chromatograms of effluent water and sludge samples taken through the pulse-fed sequencing batch reactor experiments.



1 mg/L-Effluent Water | 1 mg/L-Sludge

10 mg/L-Effluent Water | 10 mg/L-Sludge

20 mg/L-Effluent Water | 20 mg/L-Sludge

50 mg/L-Effluent Water | 50 mg/L-Sludge

100 mg/L-Effluent Water | 100 mg/L-Sludge

Figure 12 HPLC-UV chromatograms of TCS analysis in effluent water and sludge samples of the reactor (Influent TCS = 1, 10, 20, 50 and 100 mg/L, acclimated culture)

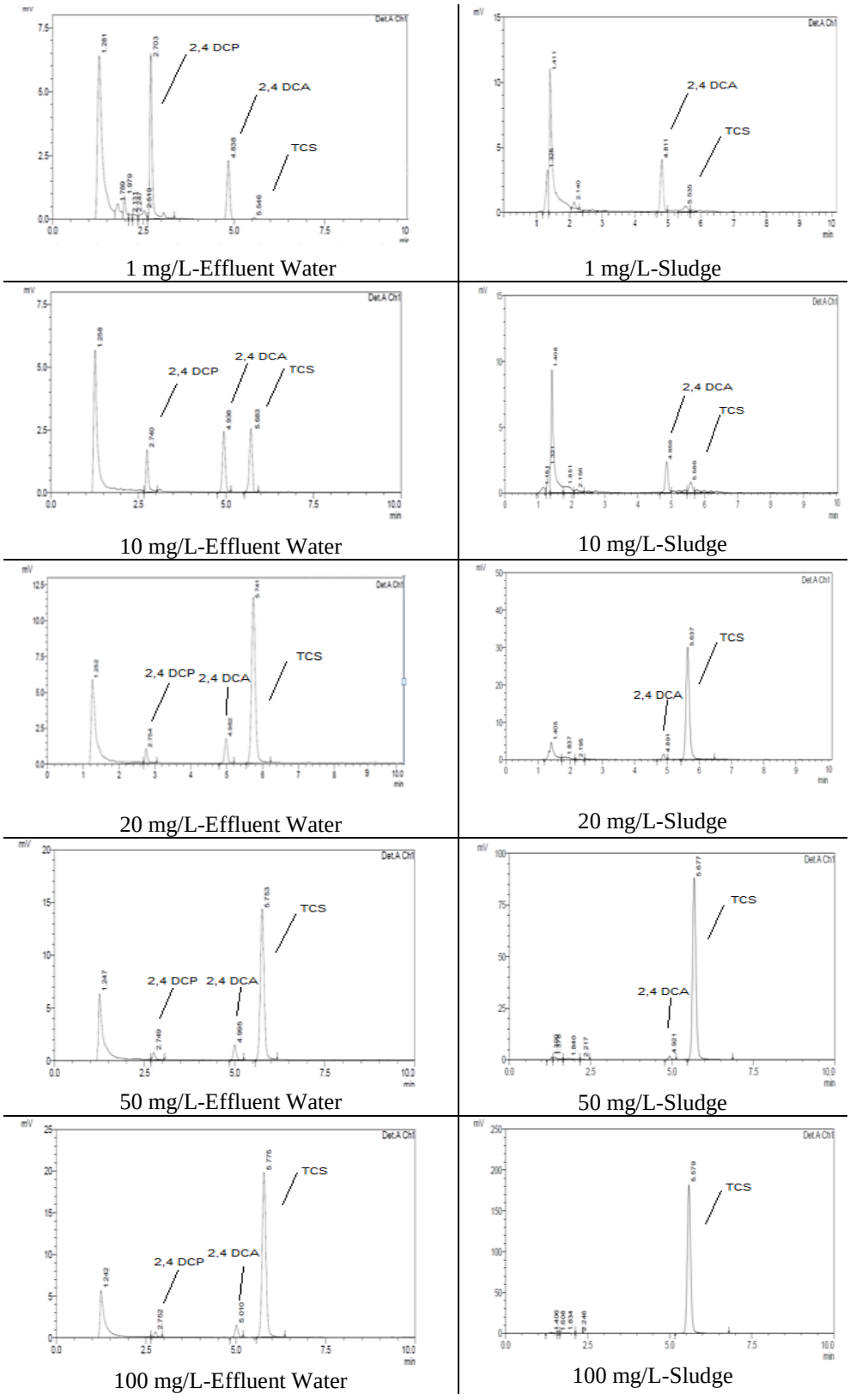


Figure 13 HPLC-UV chromatograms of TCS analysis in effluent water and sludge samples of the reactors (Influent TCS = 1, 10, 20, 50 and 100 mg/L, non-acclimated culture)

The retention time of the bought standards, the time of which equals the detection of corresponding peaks in the chromatograms, were determined as 2.6-2.7 min for 2,4-DCP, 1.9-2 min for 4-CC, 1.85 min for 4-CR and 4.9-5 min for 2,4-DCA, respectively.

The peaks observed at 2.7 min and 4.9 min in the chromatograms represented in Figure 12 and Figure 13 were coincided with the peaks of 2,4-DCP and 2,4-DCA as illustrated in Figure 14. These results proved that 2,4-DCP and 2,4-DCA were the biodegradation by-products of TCS in this study. Descriptive information regarding these by-products is provided in Appendix B.

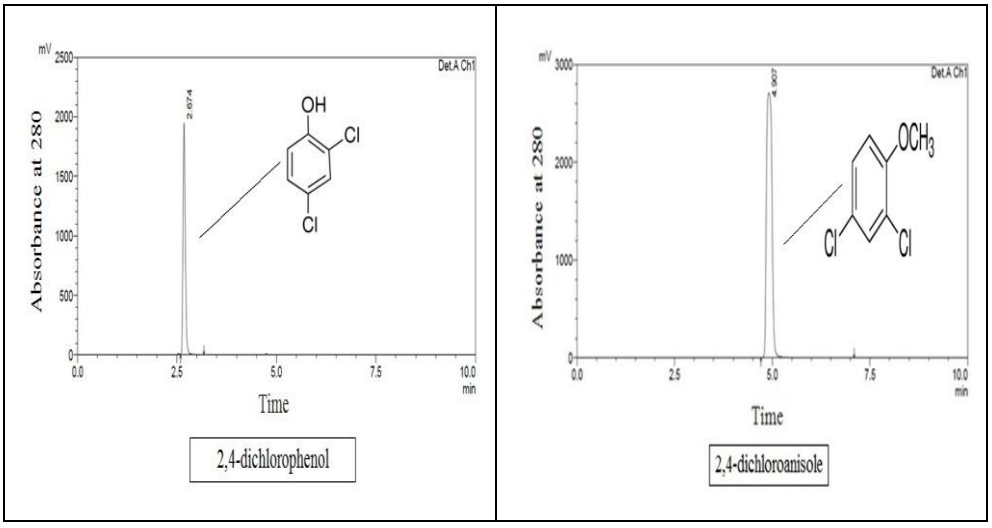


Figure 14 HPLC-UV chromatograms of 2,4-DCP and 2,4-DCA standards

When the situations inducing formation of the aforementioned by-products were of consideration, it was clearly seen that 2,4-DCP was observed all in the effluent water samples taken from the acclimated culture operating reactor except for the influent TCS concentration of 1 mg/L. For the acclimation with 1 mg/L TCS concentration, none of input TCS was remained in the liquid phase and only 2,4-DCA was detected in the effluent water sample. On the other hand, 2,4-DCP was formed as a metabolite and detected in each sludge samples taken from the acclimated culture operating reactor excluding the case of 100 mg/L TCS input. In this respect, there was

a strong possibility that TCS can transform into 2,4-DCP at the end of biological treatment when the acclimated microbial culture was exposed to TCS and this metabolite can finally end up in effluent water and sludge phase and there was a slight probability to find 2,4-DCA as a biodegradation metabolite of TCS in the effluent water.

In other respects, when the by-products formed in the pulse-fed sequencing batch reactors performed without biomass acclimation were evaluated, 2,4-DCP and 2,4-DCA were identified in all effluent water samples and 2,4 DCA was detected in whole sludge samples apart from the influent TCS concentration of 100 mg/L. According to these findings, it can be concluded that 2,4-DCP and 2,4-DCA are the potential biotransformation products of TCS in the situations when the TCS non-acclimated systems were experienced instantaneous TCS input.

The concentrations of the aforesaid by-products in effluent water and sludge samples are summarized in Table 12. As can be seen from this table, the metabolites were detected more in concentration in the samples belonging to non-acclimated cultures when compared to the samples of acclimated cultures. There was a decreasing trend in the observed by-product concentration following the increase in the influent TCS concentration. 2,4-DCA was not identified on the effluent water or sludge samples of acclimated culture (excluding 1 mg/L initial TCS concentration). On the other hand, the higher 2,4-DCA concentrations were observed both in effluent water and sludge samples of non-acclimated cultures. Furthermore, there was no sludge accumulation of 2,4-DCP for the non-acclimated cultures whereas it was found around 10-60 μg/L in effluent water samples based upon the initial TCS concentration fed to the reactors.

When these findings were evaluated in general, the positive effect of acclimation on biodegradation metabolite formation was definitely seen and

it can be stated that after certain acclimation period biodegradation rather than biotransformation was the main mechanism involved in TCS removal.

Table 12 By-product concentrations for acclimated and non-acclimated cultures

	2,4 DCP (µg/L)		**2,4 DCA (µg/L)**	
Sample –TCS (mg/L)	**Effluent Water**	**Sludge**	**Effluent Water**	**Sludge**
Acclimated culture-1	-	-	90.66	-
Acclimated culture -10	6.61	8.24	-	-
Acclimated culture -20	4.73	5.28	-	-
Acclimated culture -50	4.95	4.98	-	-
Acclimated culture -100	4.47	-	-	-
Non-acclimated culture -1	62.73	-	153.14	277.50
Non-acclimated culture -10	19.88	-	160.26	168.57
Non-acclimated culture -20	13.22	-	112.93	100.19
Non-acclimated culture -50	12.09	-	95.97	134.83
Non-acclimated culture -100	10.07	-	96.80	-

TCS and its aerobic biodegradation by-products are compared in Table 13 considering their physical and chemical properties and assosicated risk phrases, and assessments were done according to the data provided in this table. The description of the risk phrases is given in Appendix C.

Table 13 Comparison of TCS and its biotransformation products based on their physical and chemical properties (NICNAS, 2009; EU SCCP, 2010; Ciba-Geigy Limited, 1990f; OECD SIDS, 2006; URL 1; URL 2)

Parameter	TCS	2,4-DCA	2,4-DCP
CAS Number	3380-34-5	553-82-2	120-83-2
Log K_{ow} or Log P_{ow} at 20°C	4.76	-	3.21-3.25
Solubility at 20°C (mg/L)	1	-	4500
pKa	8.14	-	7.89
Vapor pressure at 25°C (Pa)	7×10^{-4}	-	16
BCF	8700 (Zebra fish)	-	69 (Carp)
EDC	Most probably	-	Ambiguous
Half life in stream water (day)	>365	-	30
NOEC (mg/L)	0.0005 (for algae)	-	0.21 (Daphnia Magna)
Risk phrases	R23 R36 R37 R38 R41 R50/53	R36/37/38 R20/22	R21/22 R34 R51/53

As can be seen from Table 13, 2,4-DCP, one of the by-products of TCS, is less toxic, bio-accumulative and persistent than the parent chemical. Additionally, 2,4-DCP is more volatile and lipophilic due to its having higher vapor pressure and higher solubility in water when compared to those of TCS, respectively. In this sense, it can be said that this biological

transformation product is less dangerous than the parent compound when the chemical properties and associated risk status of them are sought thoroughly. In other words, the presence of 2,4-DCP in water environments can be ignored up to a certain toxic concentration which does not constitute any problem to the most sensitive aquatic organism whereas the bioavailability of TCS posses more risks to the surrounding environment and that is why it should be dealed in the prior stage when both chemicals exist in the water body at the same time.

On the other hand, no exact conclusion can be made regarding the other by-product, namely, 2,4-DCA since there exists no adequate information on the physical and chemical properties of this compound in the literature. Only data obtained from the literature is about the risk phrases of 2,4-DCA and when the risks associated with this substance are compared with those of TCS, it can be stated that TCS has a more potential of adverse effects on the aquatic ecosystem and thus the presence of it might be more risky in the water bodies of concern.

After considering the findings of by-product and mass balance analyses for the acclimation and non-acclimation cases and relating them to those of the pulse-fed sequencing batch reactor operations, biological treatment performance of the system in terms of COD removal can be more easily interpreted. In this respect, it should be noted that in spite of the fact that the obtained effluent COD values (35-263 mg/L for the acclimation case; 170-289 mg/L for the non-acclimation case) for the pulse-fed sequencing batch reactors were a little bit contributed by the COD equivalence of TCS and its by-products, the mass balance and by-product analysis result showed that the steady state concentrations of those were low (0-14.160 mg/L, 0-0.091 mg/L and 0-0.007 mg/L for TCS; 2,4-DCA and 2,4-DCP respectively for the acclimation case; 0.020-10.530 mg/L, 0.096-0.160 mg/L and 0.010-0.063 mg/L for TCS; 2,4-DCA and 2,4-DCP respectively for the non-acclimation case) in the liquid phase after treatment and therefore, COD

contribution by them can be ignored when compared to that of peptone. Thus, the achived COD removal efficiencies can be considered as system treatment performance in terms of peptone.

4.2 Kinetics Studies

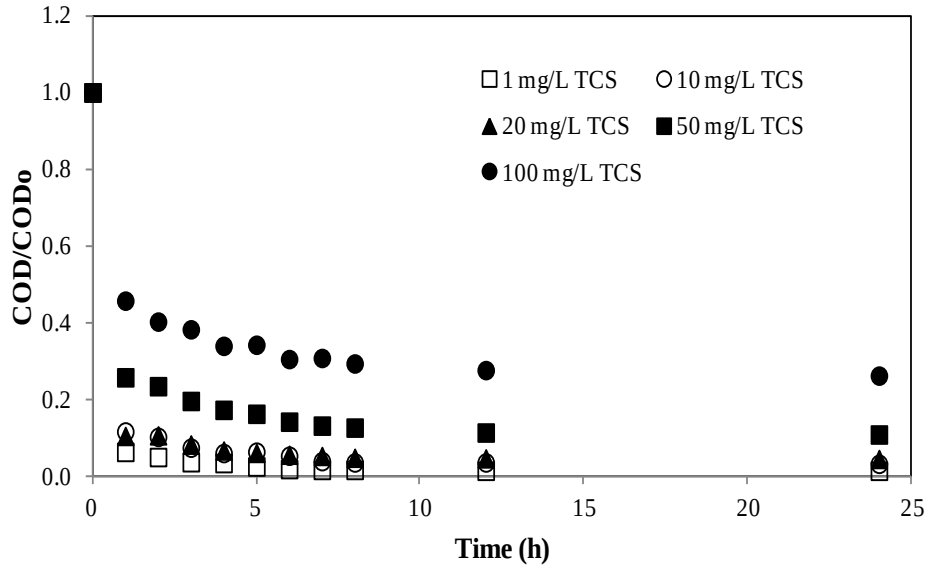
Initial biomass concentration (minimum 215 mg/L) was kept as much as possible during the batch reactor operations in order to accelerate the degradation, decrease the acclimation period (lag phase) of microbial culture to the environmental conditions and avoid them to be exposed to the additional adaptation, keep the change in biomass concentration at minimum and thus provide the observed kinetic constants not to be affected from the existing situation anymore.

The aforementioned minimum initial biomass concentration corresponded to the value less than 0.28 of TCS/biomass ratio on COD basis (<0.28) for the acclimated culture experiments and less than 0.36 of TCS/biomass ratio on COD basis (<0.36) for the non-acclimated culture experiments.
Within the scope of batch reactor operations, the peptone degradation was studied on raw sludge primarily in the absence of TCS. By this way, the yield coefficient (Y, g MLVSS/g substrate consumed) of the system, namely the amount of solid material produced per unit of substrate removed, was investigated through TCS addition. The yield coefficient of the control reactor operated in TCS-free environment was found as 0.3488 g MLVSS/g COD.

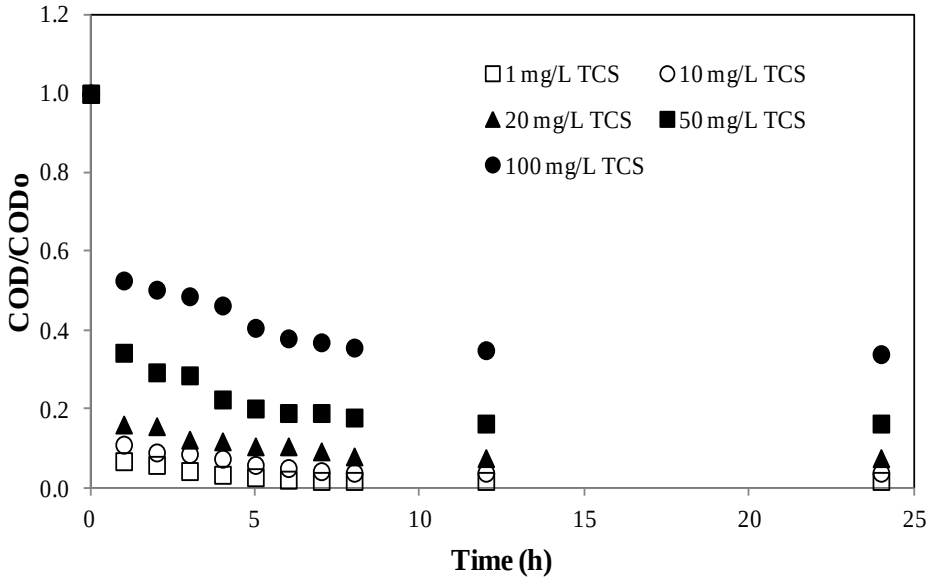
4.2.1 TCS Removal Kinetics in the Presence of Peptone

Batch reactor operations were performed in the presence of readily biodegradable substrate source that was peptone in this study and TCS as a secondary substrate source for both acclimated and non-acclimated culture environments. The obtained results were taken into consideration below.

Time dependent COD variations of the batch reactors operated in the peptone bearing environment for acclimated and non-acclimated cultures are illustrated in Figure 15.



(a)



(b)

Figure 15 Time dependent COD variations of the batch reactors operated in the presence of peptone for acclimated (a) and non-acclimated (b) cultures

As can be seen from Figure 15, there was a sharp decrease in COD values during initial 1 h for both acclimated and non-acclimated cultures including

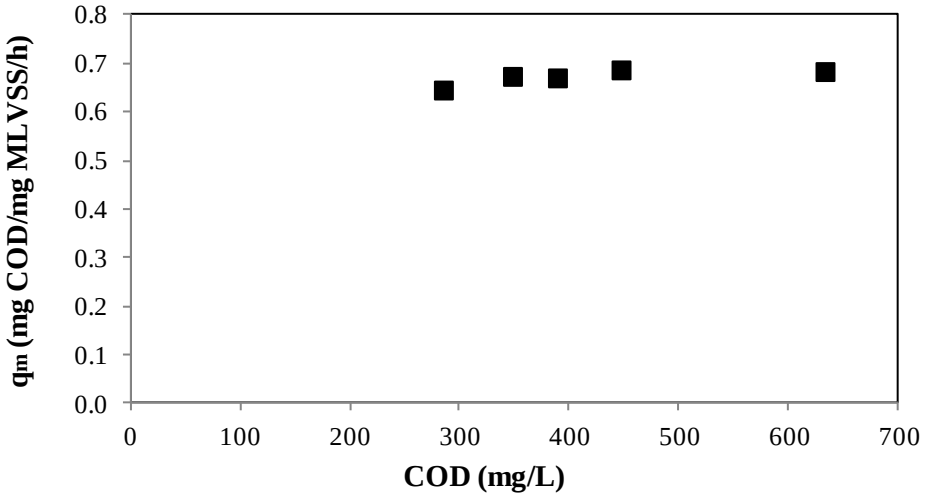
reactors. COD removal efficiencies of the systems were reduced with the increased TCS concentration (Table 14). This situation showed parallelism in terms of the trend observed with the outcomes of pulse-fed sequencing batch reactors in spite of the different removal percentage values obtained due to the differences involved in the operation conditions. It was clear that TCS had no effect on the peptone use by the microorganisms for concentration between 1 mg/L and 20 mg/L.

On the other hand, COD removals were a bit more inhibited by the influent contaminant concentrations above 50 mg/L and TCS adversely affected the use of readily biodegradable peptone when it was existing at these concentrations. Although the COD removal efficiency was decreased by the presence of TCS, maximum specific substrate (COD) utilization rate (q_m) did not change considerably in the presence of TCS and the average maximum specific substrate (COD) utilization rate was found independently from TCS concentration as 0.682 ± 0.026 mg COD/mg MLVSS.hour and 0.683 ± 0.031 mg COD/mg MLVSS.hour for acclimated and non-acclimated cultures, respectively.

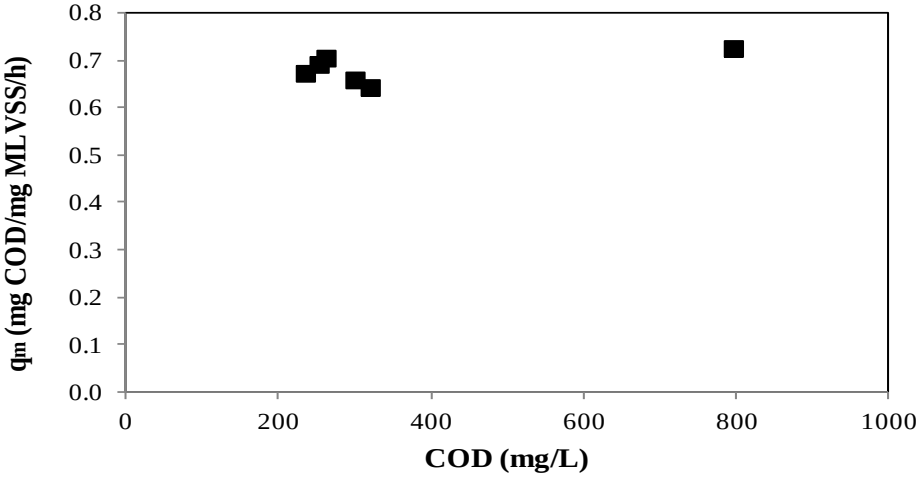
Variation in the maximum specific substrate (COD) utilization rate based on the initial substrate concentration is demonstrated in Figure 16.

Table 14 COD removal of the batch reactors in the presence of peptone

TCS, mg/L	**COD Removal, % (Acclimated)**	**COD Removal, % (Non-acclimated)**
0	99	99
1	99	98
10	96	96
20	95	92
50	89	84
100	72	66



(a)

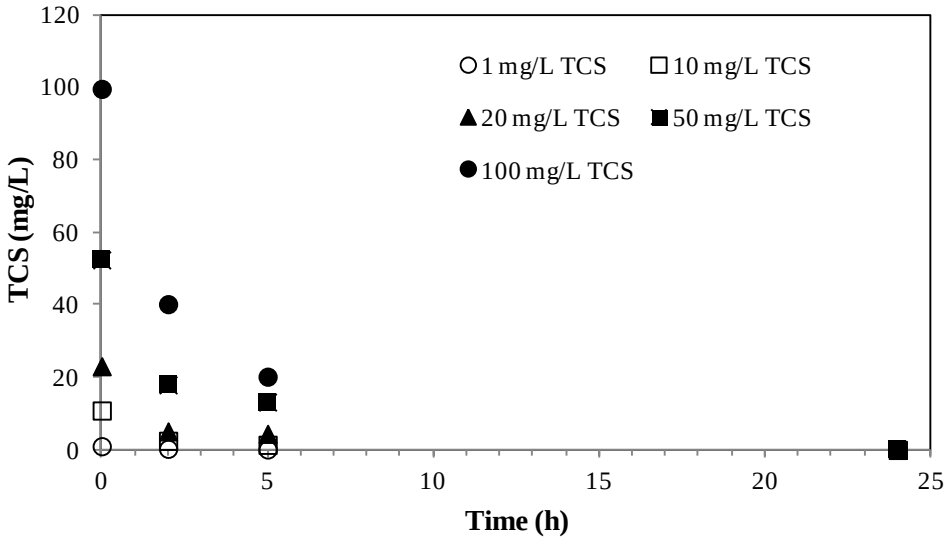


(b)

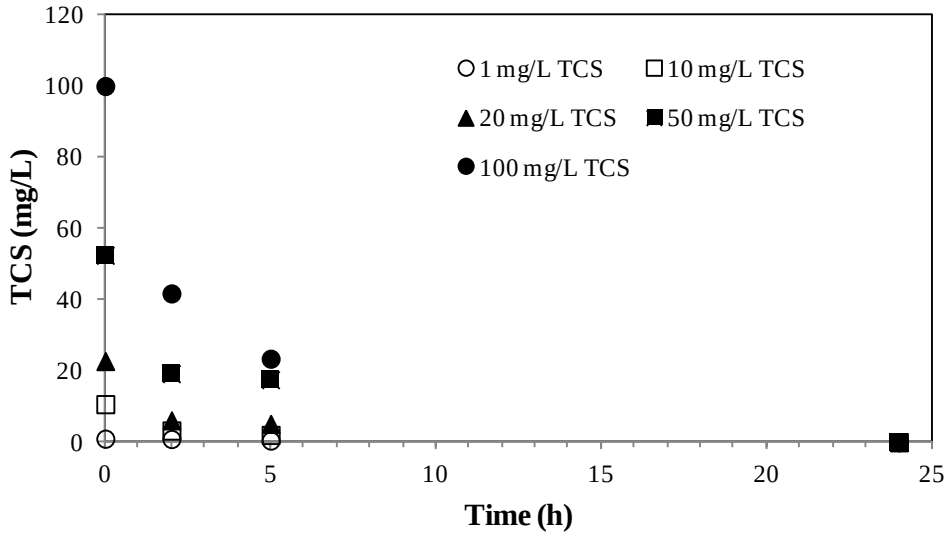
Figure 16 Maximum specific substrate (COD) utilization rate (q_m) corresponding to the initial substrate concentration for the batch reactors operated in the presence of peptone for acclimated (a) and non-acclimated (b) cultures

Time dependent changes in TCS concentration observed in the batch reactors are represented in Figure 17. From that figure, it is seen that TCS was completely removed from the water environment for all studied contaminant concentrations both with acclimated and non-acclimated culture at the end of 24 h. As expectedly, time required for the same TCS removal effiency in the system was increased with the higher TCS input to the batch reactor. There was observed a dramatic decrease in TCS

concentrations in first 2 h and TCS removal was almost completed at the end of 5 h. Decreased removal rate of TCS after first 2 h was considered as an indicator of the adsorption of TCS by the biomass and thereafter its metabolization by that means. This situation was complied with the hydrophobic characterici ty of TCS and the findings of the pulse-fed sequencing batch reactor operations mentioned in the above sections.



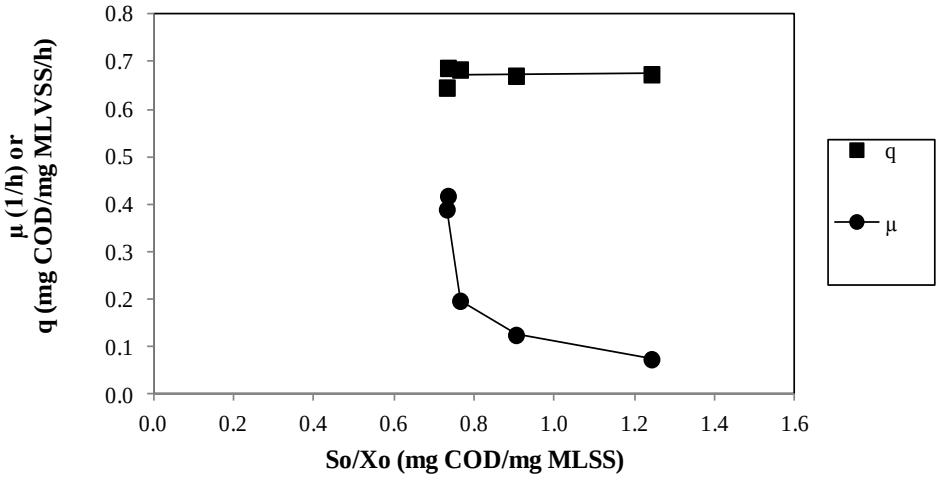
(a)



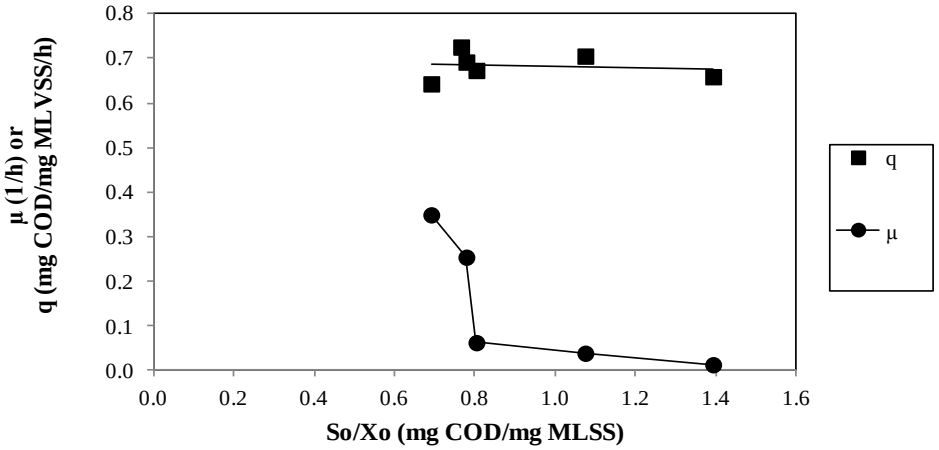
(b)

Figure 17 Time dependent changes in TCS concentration of the batch reactors operated with acclimated (a) and non-acclimated (b) cultures in the presence of peptone

Figure 18 demonstrates the variation of the specific growth rate (μ) with the initial substrate/biomass ratio (S_o/X_o). Neglecting the fact that the calculated μ values did not entirely represent the substrate metabolism due to the adsorption of TCS by the biomass at the beginning, it can be said that the decrease in μ values with the increased S_o/X_o ratio was an indicator of TCS inhibitory effect on the readily biodegradable substrate (peptone) utilization. To support this argument, Figure 19 is prepared so as to illustrate the variation of q, μ and Y values according to TCS concentration. As depicted from this figure, initial q values remained unchanged with TCS concentration whereas μ and Y values got changed remarkably. There was observed an increase in μ and Y values for TCS concentrations of 1 mg/L and 10 mg/L when compared to the values obtained in the absence of TCS. However, the increased trends in the aforesaid values did not sustained so long and μ and Y values decreased rapidly at TCS concentration above 10 mg/L. This can be interpreted as that TCS had a stimulant effect on the peptone utilization at low concentrations. The decrease in Y values with the increased TCS concentrations was an evidence of biomass tending towards to the endogeneous metabolism (maintenance) from the sytnhesis metabolism (cell growth).

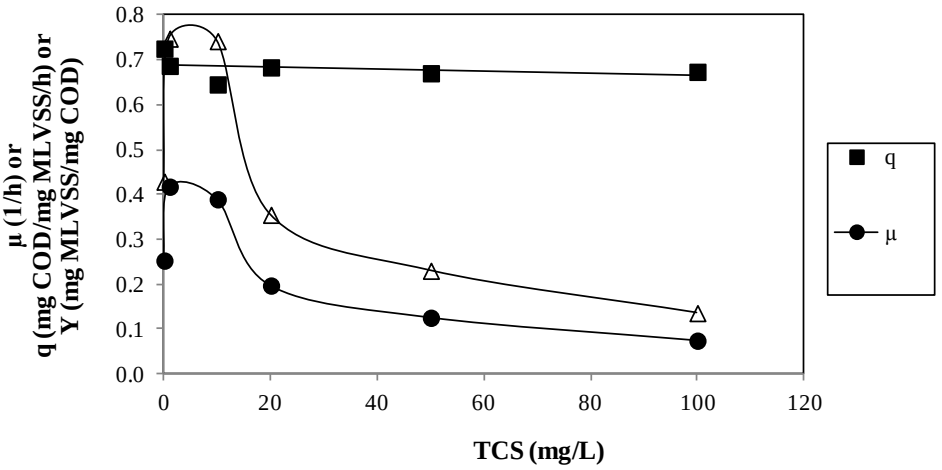


(a)

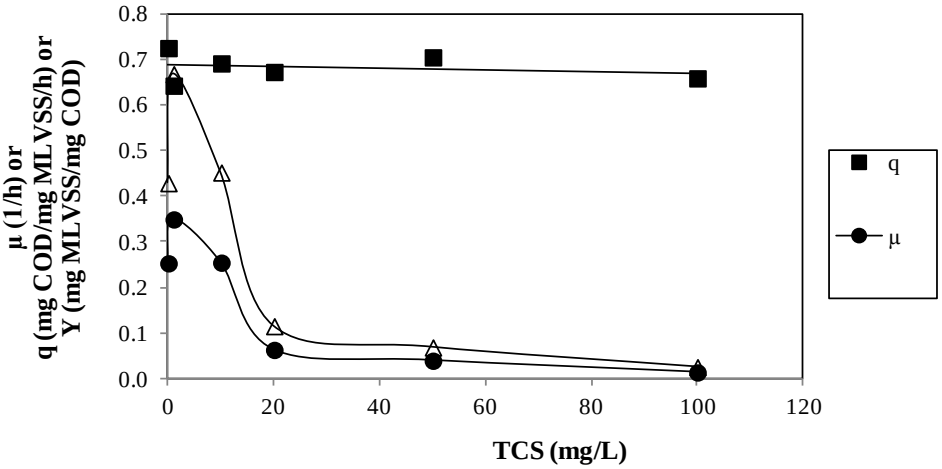


(b)

Figure 18 Variation of μ and q values with the initial S_o/X_o value for the batch reactors operated with acclimated (a) and non-acclimated (b) cultures in the presence of peptone



(a)



(b)

Figure 19 Variation of μ, q and Y values with the initial TCS concentration for the batch reactors operated with acclimated (a) and non-acclimated (b) cultures in the presence of peptone

Specific TCS removal rates of the acclimated and non-acclimated cultures are given in Figure 20 comparatively. As seen from Figure 20, specific TCS removal rate was directly proportional to TCS concentration and it was increasing with the increased TCS concentration no matter what the nature of the microbial culture (i.e. acclimated or non-acclimated culture) was involved in the reactor.

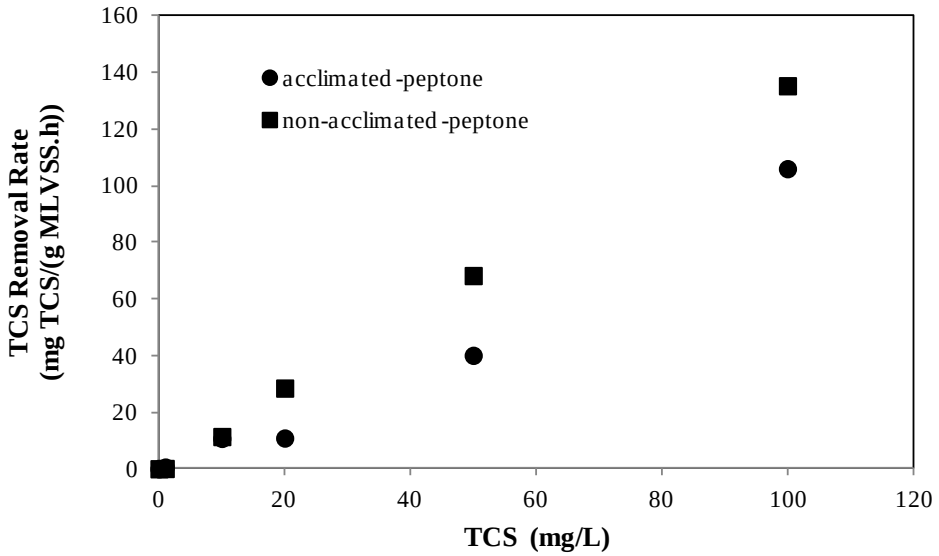
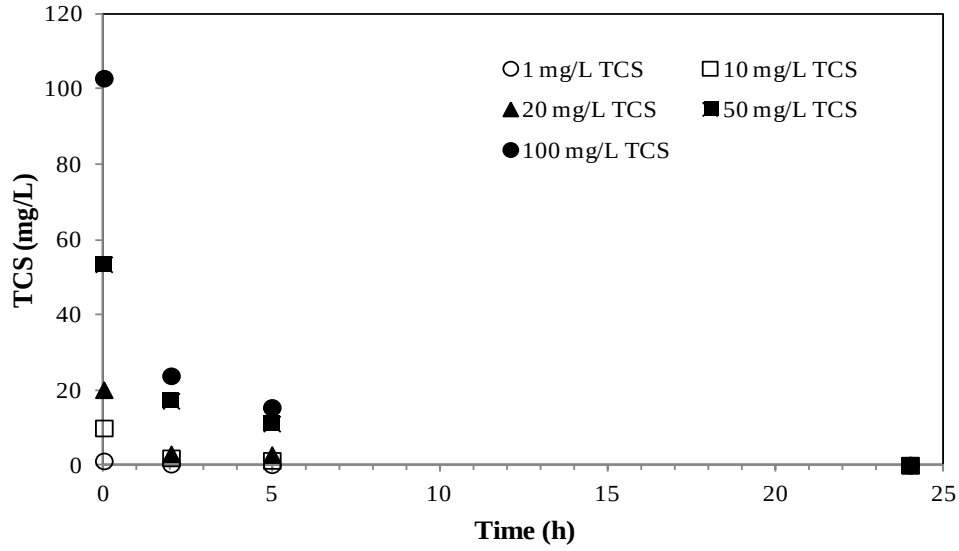


Figure 20 Variation of specific TCS removal rate with the influent TCS concentration for the batch reactors operated with acclimated and non-acclimated cultures in the presence of peptone

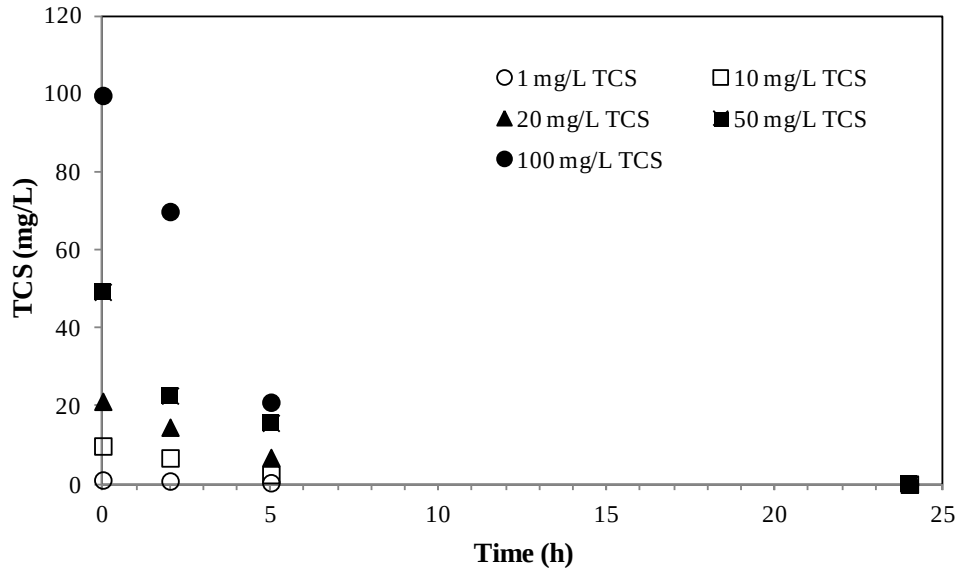
4.2.2 TCS Removal Kinetics in the Peptone-Free Environment

Batch reactor operations were performed for both acclimated and non-acclimated culture mediums in the peptone free environment, in other words, in the presence of TCS as an only substrate source. The gathered results were handled in below figures and tables.

Time dependent TCS variations of the batch reactors operated in the peptone free environment for acclimated and non-acclimated cultures are represented in Figure 21.



(a)



(b)

Figure 21 Time dependent TCS variations of the batch reactors operated in the peptone free environment for acclimated (a) and non-acclimated (b) cultures

As in the case of the experiments conducted in the presence of peptone, it was also observed in the peptone free environment that TCS was completely removed from water for all studied contaminant concentrations both with acclimated and non-acclimated culture at the end of 24 h. At the same time, TCS removal rate was observed to be different based on the acclimation condition in the peptone free environment as opposed to the ones acquired

in the peptone bearing environment. It is seen from Figure 21 that biomass acclimation affects TCS removal in a positive manner and accelerates the removal of this contaminant from the water environment. For instance, a significant portion of TCS removal was occurred in the first 5 h for the reactors operated with non-acclimated culture while the same removal efficiency was observed in first 2 h for the acclimated culture including reactors. This situation points out the importance of the acclimation in the peptone free environment in contrast to peptonic medium.

Specific TCS removal rates of the acclimated and non-acclimated cultures are illustrated in Figure 22 comparatively. As depicted from the figure, specific TCS removal rate was increasing directly with the increased TCS concentration no matter what the nature of the microbial culture (i.e. acclimated or non-acclimated culture) was involved in the reactors for TCS concentrations up to 50 mg/L. However, specific TCS removal rate was observed substantially higher for the batch reactors bearing acclimated cultures (i.e. nearly 3 times higher) rather than non-acclimated cultures above contaminant concentration of 50 mg/L.

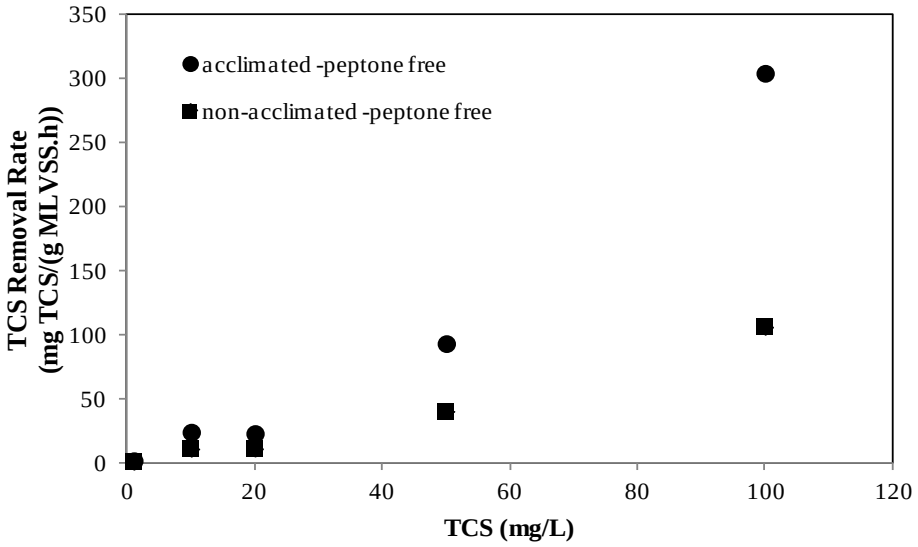
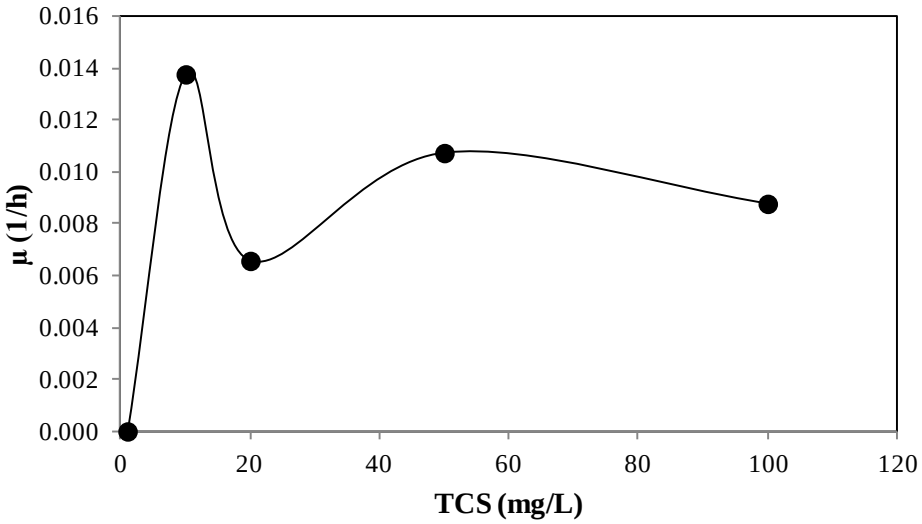
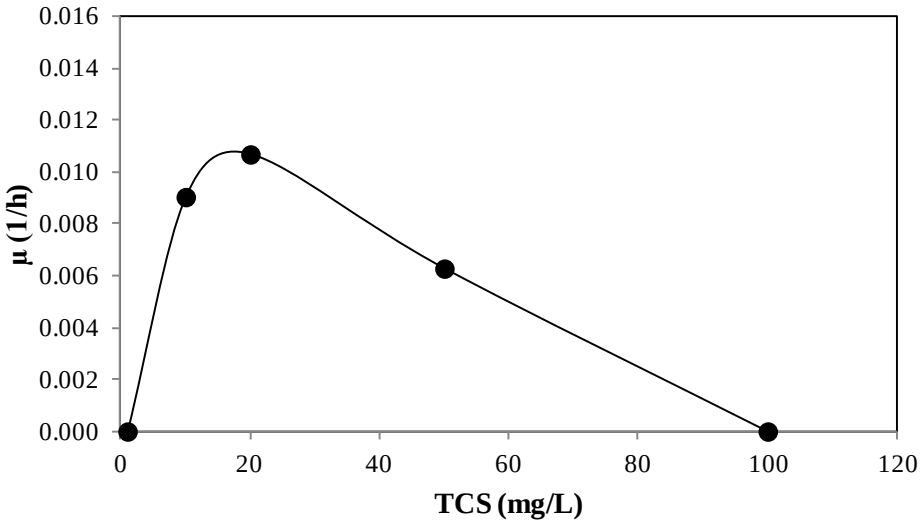


Figure 22 Variation of specific TCS removal rate with the influent TCS concentration for the batch reactors operated with acclimated and non-acclimated cultures in the peptone free environment

The variation of μ values according to TCS concentration is represented in Figure 23 for the batch reactors operated with acclimated and non-acclimated cultures.



(a)



(b)

Figure 23 Variation of specific growth rate with the influent TCS concentration for the batch reactors operated with acclimated (a) and non-acclimated (b) cultures in the peptone free environment

As illustrated in Figure 23, the pattern of the graph obtained for non-acclimated culture case was an indicator of substrate inhibition. Increase of

TCS concentration above 20 mg/L was resulted in the decrease of μ values and the microbial synthesis was wholely ceased at a contaminant concentration of 100 mg/L. On the other hand, TCS removal was still observed at a concentration of 100 mg/L which demonstrating the transition of the endogeneous metabolism (maintenance) from the biosynthesis metabolism explicitly. K_s and K_I values for the non-acclimated culture experiments were said to be around 5 mg/L and 60 mg/L (Figure 23 b), respectively. For the acclimated culture, the inhibition effect was reduced and substrate inhibition was in an approach to the Monod Model (Figure 23 a). However, the deviation or the fluctuation obtained at 20 mg/L TCS concentration was not clearly understood.

4.3 Microbial Composition

The findings, obtained at the end of the PCR and DGGE techniques that were applied for the sludge samples following the steady state conditions at each influent TCS concentration within the scope of the pulse-fed sequencing batch reactors operated with raw sludge (free of TCS), acclimated (100 ng/L-100 mg/L TCS) and non-acclimated cultures (1-100 mg/L TCS) to an ultimate aim of microbial composition designation, are provided in Figure 24-26.

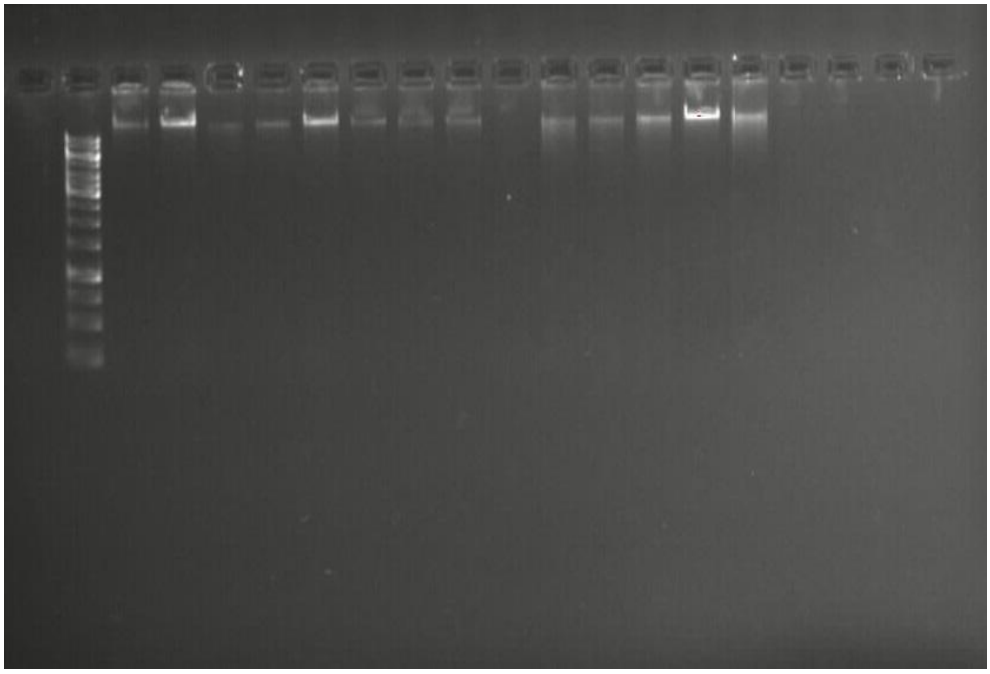


Figure 24 Genomic DNA (Gel electrophoresis image after extraction)

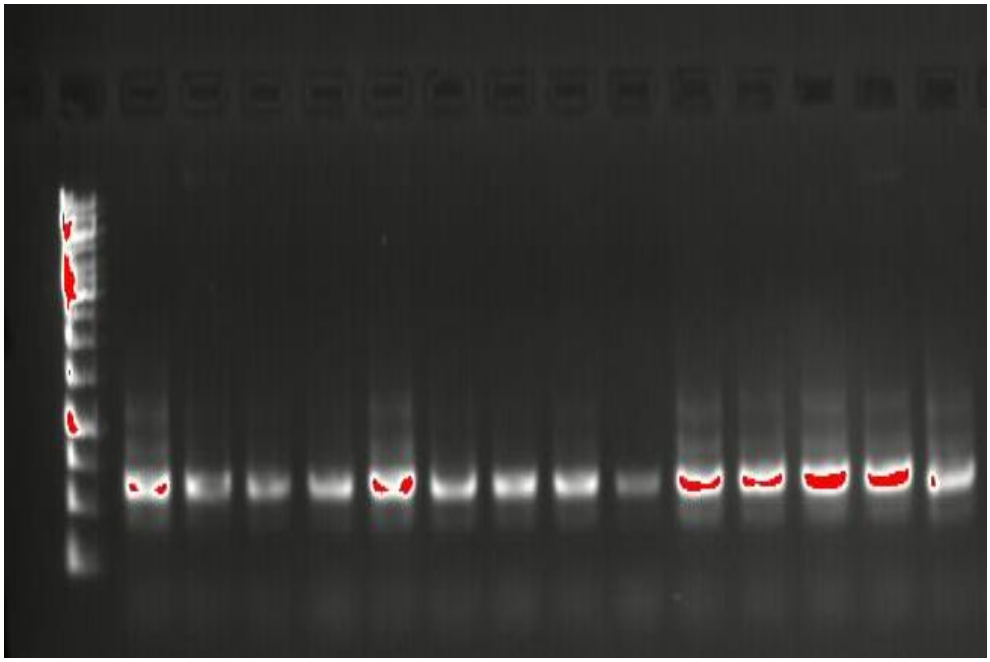


Figure 25 Gel electrophoresis image of the regarding DNA segment after PCR

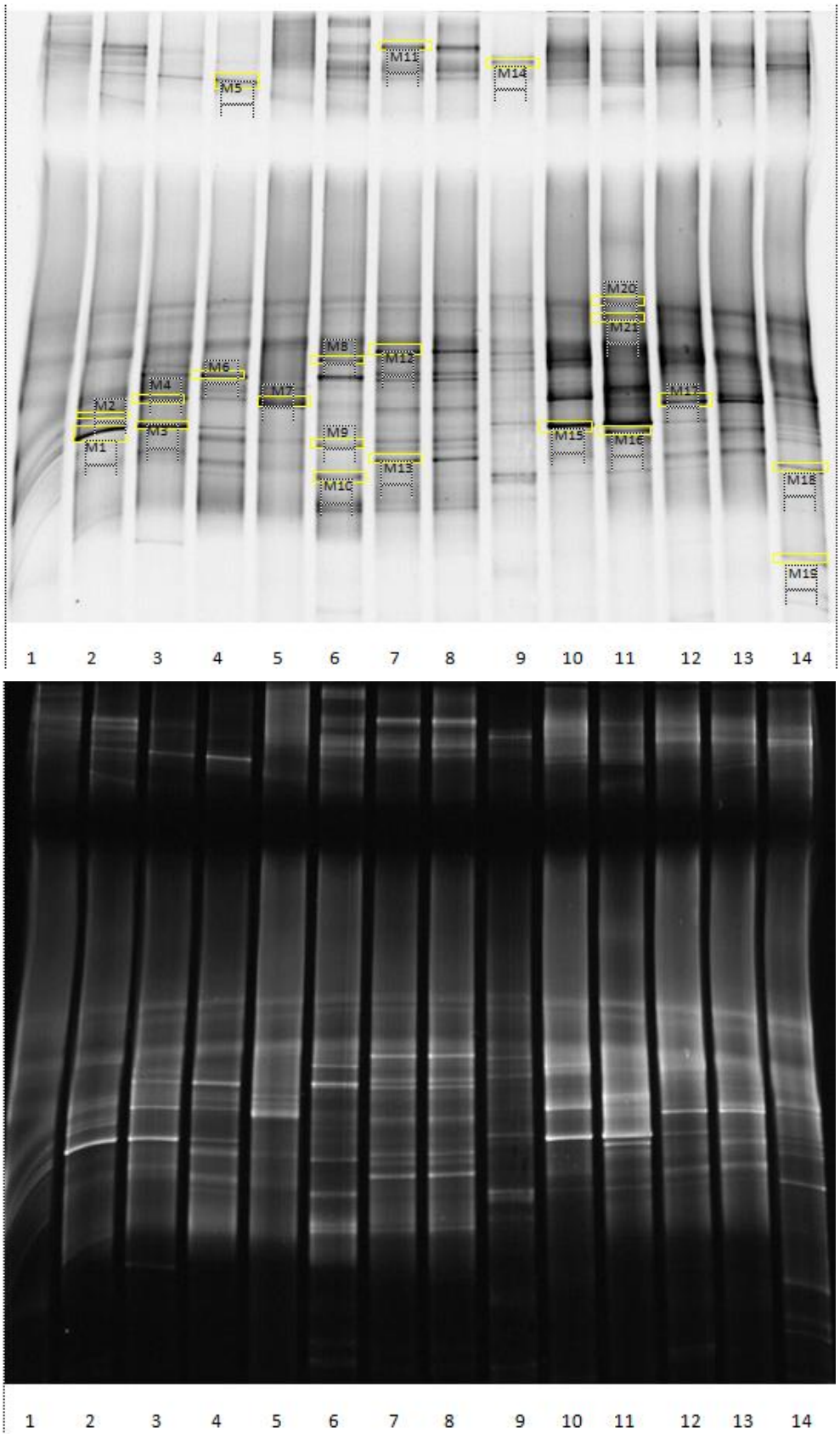


Figure 26 Gel electrophoresis image of the bands excised from DGGE gel after PCR

The excised bands were symbolized as M1, M2 and so on, and they are demonstrated in Figure 26. In the same figure, the numbers were included below the bands and each number was standed for the sludge sample dealed in the study. The sludge samples associated with these numbers included in the gel electrophoresis image of the bands excised from DGGE gel after PCR are given in Table 15. As can be seen from Figure 26, there observed no marked band in some of the sludge samples (i.e. sludge sample 1, 8 and 13); however, this situation did not mean that designation of microbial composition was not conducted for these sludge samples. Since denaturation occurs based upon the guanines (G) and cytosines (C) content of DNA sequence in DGGE technique, the bands in the same line belong to the same bacterial specie. For that reason, there is no need to excise all bands for each sample and if any of the bands is excised and identified for one sample and if it is observed to exist in any other samples in the same line, it should be considered that bacterial species designated in this band are also involved in the other samples. For instance, M20 band was excised from the one place and idendified in the certain line; however, it was apparent and present in all sludge samples although it was not excised in all samples.

Table 15 Sludge samples associated with the numbers included in the gel electrophoresis image of the bands excised from DGGE gel after PCR

Number	Sludge Sample
1	Acclimated culture -1 mg/L TCS
2	Acclimated culture -10 mg/L TCS
3	Acclimated culture -20 mg/L TCS
4	Acclimated culture -50 mg/L TCS
5	Acclimated culture -100 mg/L TCS
6	Acclimated culture -100 ng/L TCS
7	Acclimated culture -100 µg/L TCS
8	Acclimated culture -500 µg/L TCS
9	Non-acclimated culture -1 mg/L TCS
10	Non-acclimated culture -10 mg/L TCS
11	Non-acclimated culture -20 mg/L TCS
12	Non-acclimated culture -50 mg/L TCS
13	Non-acclimated culture -100 mg/L TCS
14	TCS-free control reactor

After sequence analysis, determination of microbial composition was carried out by BioEdit program for the excised bands (i.e. M1, M2 and so on) in the sludge samples associated with the aforementioned numbers and the corresponding Blast results are provided in Appendix D. As can be seen from the Blast results, microbial composition of the M12 band was not designated due to the existence of more bands in the excised one band and therefore existence of many different species accordingly. During the sequence analysis, peak is obtained for more than one base at the same time and DGGE denaturation rate changes in the range of 20-80%. In some circumstances, the bands whose G-C sequences are too close are in juxtaposition and seen as single band. In order to distinguish the aforesaid bands, it is crucial to investigate this region through a lot narrower gel intensity, in other words, it is necessary to prepare a gel of intensity as 30-40% or 75-80% and look at only the band of concern with that gel of intensity. However, it is not possible to work on the mentioned gel intensities in Bioengineering Department of Istanbul Medeniyet University, where designation of microbial composition was accomplished in the scope of this study, and that is why M12 band could not be identified in terms of its own microbial population.

As depicted from Appendix D, BioEdit program can determine the microbial community up to a chromosome basis. In order to provide the interpretability of the results and see the variation of microbial composition in the sludge samples which were exposed to TCS in different conditions (i.e. through certain acclimation period and without acclimation period) and different concentrations, the assessment was made based upon the specie basis (i.e. first name) and the obtained results are summarized in Table 16. As can be seen from this table, 106 microorganisms were detected in total on a microbial specie basis. Among these, 31 species were observed both in raw sludge sample and all TCS-exposed sludge samples no matter the acclimation and non-acclimation conditions. Also, it was found that 24 species observed in the non-acclimated cultures were not detected in the

acclimated cultures and they were eliminated from the environment. On the other hand, 5 species that were not encountered in non-acclimated cultures were survived in the acclimated cultures. However, detection of some microbial species in non-acclimated cultures at high TCS concentrations rather than low contaminant concentrations in the same condition and non-observation of some species in acclimated cultures even in low TCS inputs although they were recognized in non-acclimated cultures exposed to high amount of contaminant were not fully understood.

Potential reasons and gathered inferences are addressed in below paragraphs in detail.

Table 16 Microbial composition of sludge samples

		Acclimated Culture									Non-acclimated Culture				
	TCS (mg/L)	0	0.0001	0.1	0.5	1	10	20	50	100	1	10	20	50	100
Band	**Microbial Composition**														
M1,M2, M3,M5, M6,M20,M21*	*Comamonas sp. (M9)*	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Acidovorax sp. (M9, M10)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Alicycliphilus sp. (M9)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Ramlibacter sp. (M9)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Methylibium sp. (M9, M10)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Delftia sp. (M9)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Rubrivivax sp. (M9, M10)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Leptothrix sp. (M9, M10)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Rhodoferax sp. (M9)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Burkholderiales sp. (M9, M10)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Variovorax sp. (M9)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Verminephrobacter sp. (M9)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Polaromonas sp. (M9)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Azoarcus sp.	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Burkholderia sp. (M10, M15)	√	√	√	√	√	√	√	√	√	√	√	√	√	√

Table 16 Microbial composition of sludge samples (Cont'd)

		Acclimated Culture									**Non-acclimated Culture**				
	TCS (mg/L)	0	0.0001	0.1	0.5	1	10	20	50	100	1	10	20	50	100
Band	**Microbial Composition**														
M4	*Aeromonas sp. (M7, M17)*	√	√	√	√	√	√	√	√	√		√	√	√	√
	Cronobacter sp.	√				√	√	√	√						
	Shewanella sp. (M7, M17)	√	√	√	√	√	√	√	√	√		√	√	√	√
	Klebsiella sp.	√				√	√	√	√						
	Pseudomonas sp. (M13)	√		√	√	√	√	√	√	√				√	√
	Enterobacter sp.	√				√	√	√	√						
	Yersinia sp.	√				√	√	√	√						
	Citrobacter sp.	√				√	√	√	√						
M7,M17**	*Pseudoalteromonas sp.*	√	√	√	√					√		√	√	√	√
	Tolumonas sp.	√	√	√	√					√		√	√	√	√
M8	*Paenibacillus sp.*	√	√	√								√	√	√	√
	Bacillus sp.	√	√	√								√	√	√	√
	Desulfitobacterium sp.	√	√	√								√	√	√	√
	Thermobacillus sp.	√	√	√								√	√	√	√
	Geobacillus sp.	√	√	√								√	√	√	√
	Brevibacillus sp.	√	√	√								√	√	√	√
	Anoxybacillus sp.	√	√	√								√	√	√	√

Table 16 Microbial composition of sludge samples (Cont'd)

		Acclimated Culture									**Non-acclimated Culture**				
	TCS (mg/L)	0	0.0001	0.1	0.5	1	10	20	50	100	1	10	20	50	100
Band	**Microbial Composition**														
M9	*Herbaspirillum sp. (M10)*	√	√	√	√				√	√	√				
	Thiomonas sp. (M10)	√	√	√	√				√	√	√				
M10	*Janthinobacterium sp. (M15)*	√	√						√		√	√	√	√	√
	Herminiimonas sp. (M15)	√	√						√		√	√	√	√	√
	Methylotenera sp. (M15)	√	√						√		√	√	√	√	√
	Nitrosospira sp. (M15)	√	√						√		√	√	√	√	√
	Gallionella sp. (M15)	√	√						√		√	√	√	√	√
	Dechlorosoma sp. (M15)	√	√						√		√	√	√	√	√
	Thauera sp. (M15)	√	√						√		√	√	√	√	√
	Methylobacillus sp. (M15)	√	√						√		√	√	√	√	√
	Candidatus sp. (M15, M19)	√	√					√	√		√	√	√	√	√
	Collimonas sp.		√						√		√				
	Dechloromonas sp.		√						√		√				

Table 16 Microbial composition of sludge samples (Cont'd)

		Acclimated Culture									**Non-acclimated Culture**				
	TCS (mg/L)	0	0.0001	0.1	0.5	1	10	20	50	100	1	10	20	50	100
Band	**Microbial Composition**														
M11	*Lacinutrix sp. (M14)*	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Aequorivita sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Flavobacterium sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Gramella sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Cellulophaga sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Zunongwangia sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Myroides sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Nonlabens sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Krokinobacter sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Maribacter sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Capnocytophaga sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Ornithobacterium sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Zobellia sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Weeksella sp. (M14)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Psychroflexus sp.	√	√	√	√	√	√	√				√	√	√	√
M12															

Table 16 Microbial composition of sludge samples (Cont'd)

		Acclimated Culture									**Non-acclimated Culture**				
	TCS (mg/L)	0	0.0001	0.1	0.5	1	10	20	50	100	1	10	20	50	100
Band	**Microbial Composition**														
M13	*Pseudoxanthomonas sp.*			√	√				√	√				√	√
	Stenotrophomonas sp.			√	√				√	√				√	√
	Xylella sp.			√	√				√	√				√	√
	Methylophaga sp.			√	√				√	√				√	√
	Methylomonas sp.			√	√				√	√				√	√
	Coxiella sp.			√	√				√	√				√	√
	Thioalkalivibrio sp.			√	√				√	√				√	√
	Methylococcus sp.			√	√				√	√				√	√
	Frateuria sp. (M19)	√		√	√				√	√	√			√	√
	Xanthomonas sp. (M19)	√		√	√				√	√	√			√	√
	Rhodanobacter sp. (M19)	√		√	√				√	√	√			√	√
M14	*Pedobacter sp.*	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Mucilaginibacter sp.	√	√	√	√	√	√	√	√	√	√	√	√	√	√
M15	*Methylovorus sp.*	√									√	√	√	√	√
	Sideroxydans sp.	√									√	√	√	√	√

Table 16 Microbial composition of sludge samples (Cont'd)

		Acclimated Culture									**Non-acclimated Culture**				
	TCS (mg/L)	0	0.0001	0.1	0.5	1	10	20	50	100	1	10	20	50	100
Band	**Microbial Composition**														
M16	*Paracoccus sp.*	√											√		√
	Ketogulonigenium sp.	√											√		√
	Rhodobacter sp.	√											√		√
	Ruegeria sp.	√											√		√
	Phaeobacter sp.	√											√		√
	Roseobacter sp.	√											√		√
	Jannaschia sp.	√											√		√
	Dinoroseobacter sp.	√											√		√
	Parvibaculum sp.	√											√		√
M18	*Clavibacter sp.*	√									√	√	√	√	√
	Leifsonia sp.	√									√	√	√	√	√
	Intrasporangium sp.	√									√	√	√	√	√
	Microbacterium sp.	√									√	√	√	√	√
	Arthrobacter sp.	√									√	√	√	√	√
	Isoptericola sp.	√									√	√	√	√	√
	Cellulomonas sp.	√									√	√	√	√	√
	Beutenbergia sp.	√									√	√	√	√	√

Table 16 Microbial composition of sludge samples (Cont'd)

		Acclimated Culture									Non-acclimated Culture				
	TCS (mg/L)	0	0.0001	0.1	0.5	1	10	20	50	100	1	10	20	50	100
Band	**Microbial Composition**														
M18	*Micrococcus sp.*	√									√	√	√	√	√
	Streptomyces sp.	√									√	√	√	√	√
	Frankia sp.	√									√	√	√	√	√
	Cellvibrio sp.	√									√	√	√	√	√
	Kocuria sp.	√									√	√	√	√	√
M19	*Caldicellulosiruptor sp.*	√						√			√				
	Terriglobus sp.	√						√			√				
	Nitrosococcus sp.	√						√			√				
	Granulicella sp.	√						√			√				
	Hippea sp.	√						√			√				
	Conexibacter sp.	√						√			√				
	Leptospirillum sp.	√						√			√				
	Acidobacterium sp.	√						√			√				
	Geobacter sp.	√						√			√				

* When the assessment was made on species basis, M1, M2, M3, M5, M6, M20 and M21 bands were completely the same.

** When the assessment was made on species basis, M7 and M17 bands were completely the same.

As can be seen from Table 16, the assigned microbial composition of different bands can be the same. When viewed from this aspect, the bands of M1, M2, M3, M5, M6, M20 and M21 were completely same on a specie basis and they may have involved common microorganisms with the other bands. To illustrate, the species detected in M1, M2, M3, M5, M6, M20 and M21 bands, namely, "*Comamonas sp., Acidovorax sp., Alicycliphilus sp., Ramlibacter sp., Methylibium sp., Delftia sp., Rubrivivax sp., Leptothrix sp., Rhodoferax sp., Burkholderiales sp., Variovorax sp., Verminephrobacter sp., Polaromonas sp.*",were also encountered in M9 band at the same time. Similarly, M7 and M17 bands were representing the same microbial community and therefore they were entirely the same.

M14, M20 and M21 bands were recognized in the sludge samples free of TCS and also acclimated and non-acclimated with TCS. In other words, the microbial community respresented with these bands were observed in all sludge samples whether TCS was present or not in the environment. The most important implication of this founding is that the microorganisms determined in the aforesaid bands are resistant and not sensitive to TCS and they can survive even in high TCS concentrations. Another inference is that TCS has no toxic effect on the microbial community of these bands in the studied concentration range (i.e.100 ng/L-100 mg/L). In any case, either acclimation or not, these microorganisms were maintained in the environment and not affected by the presence of TCS (i.e. *Pedobacter sp., Mucilaginibacter sp, Lacinutrix sp., Aequorivita sp., Flavobacterium sp., Gramella sp., Cellulophaga sp., Zunongwangia sp., Myroides sp., Nonlabens sp., Krokinobacter sp., Maribacter sp., Capnocytophaga sp., Ornithobacterium sp., Zobellia sp., Weeksella sp., Comamonas sp., Acidovorax sp., Alicycliphilus sp., Ramlibacter sp., Methylibium sp., Delftia sp., Rubrivivax sp., Leptothrix sp., Rhodoferax sp., Burkholderiales sp., Variovorax sp., Verminephrobacter sp., Polaromonas sp., Azoarcus sp., Burkholderia sp*).

The microbial communities of the M11 and M14 bands were same to a large extent. Considering the presence of M14 band in all sludge samples, the great majority of the microorganisms encountered in M11 band was also expected to be found in all sludge samples accordingly. M11 band was observed in raw sludge sample and it was also recognized in the sludge samples acclimated with 100 ng/L, 100 µg/L, 500 µg/L, 1 mg/L, 10 mg/L and 20 mg/L TCS concentration, respectively.However, the aforementioned band was not seen in the sludge samples acclimated with higher TCS concentrations (i.e. 50 mg/L and 100 mg/L); thus, apart from the microbial species common with the M14 band, the microorganism represented by this band (*i.e. Psychroflexus sp.*) could not survive despite the prior acclimation with the contaminant when it is faced with high amount of TCS and right after eliminated from the environment. For that reason, it can be said that TCS at 50 mg/L and 100 mg/L concentration is toxic to the microorganisms represented by M11 band excluding the common ones with the M14 band.

M18 and M15 bands were identified in the raw sludge sample and not observed in the sludge samples acclimated with TCS. In other respects, these bands were found in all of the sludge samples taken from the pulse-fed sequencing batch reactors fed by TCS under non-acclimation conditions (i.e. even in high contaminant concentrations). Thus, the microbial community declared with these bands can said to be the microorganisms accommodated to the environments where non-acclimation conditions and instantaneous contaminant disharges are prevailing and they are the ones dominating in such environments (i.e. *Methylovorus sp., Sideroxydans sp., Clavibacter sp., Leifsonia sp., Intrasporangium sp., Microbacterium sp., Arthrobacter sp., Isoptericola sp., Cellulomonas sp., Beutenbergia sp., Micrococcus sp., Streptomyces sp., Frankia sp., Cellvibrio sp., Kocuria sp.*).

M8 band was detected in raw sludge sample and the sludge samples acclimated with 100 ng/L and 100 µg/L TCS concentrations, as well. However, it was not detected in the acclimated sludge samples along with

500 µg/L TCS dosage and more. In other words, the microorganisms encountered in this band are resistant to 100 ng/L and 100 µg/L TCS concentration and they can survive to the presence of the contaminant at such amount, but, they are sensitive to TCS when it is available with a concentration equals and greater than 500 µg/L and concentration of 500 µg/L is adequate for the aforesaid microbial community to be eliminated from the environment (i.e. *Paenibacillus sp., Bacillus sp., Desulfitobacterium sp., Thermobacillus sp., Geobacillus sp., Brevibacillus sp., Anoxybacillus sp.*).

On the other side, some microorganisms were observed in acclimated and non-acclimated sludge samples although they were not identified in raw sludge sample (i.e. *Collimonas sp., Dechloromonas sp., Pseudoxanthomonas sp., Stenotrophomonas sp., Xylella sp., Methylophaga sp., Methylomonas sp., Coxiella sp., Thioalkalivibrio sp., Methylococcus sp.*). This situation can be explained in that way: During the analysis total DNA was extracted and then the certain fragments amplificated with PCR. Considering the presence of the species in raw sludge that are capable of degrading the chlorinated compounds, if the concentration of these kinds of species are too low, it might not be possible to detect them with DGGE. In such condition, the excised band will be so weak that it can not be seen any more. However, when the chlorinated compounds like TCS are supplied to the environment, the specie, the number of which is million-to-one of the other existing species, immediately increases in numbers and becomes dominant in the environment. Through the dominancy of this specie, the chance of it to be observed with DGGE increases directly. Therefore, the species not encountered in TCS free raw sludge sample can be detected in TCS bearing sludge samples, as well.

The species seen in M4 band, namely, Cronobacter sp., Klebsiella sp., Enterobacter sp., Yersinia sp. and *Citrobacter sp.* were detected in raw sludge and acclimated sludge samples but not recognized in the samples

including non-acclimated cultures. From this observation, it is understood that these species maintain their life under acclimation conditions when the exposure of the contaminant becomes a current issue.

According to the other study, *Staphylococci sp., Streptococci sp., Mycobacteria sp., Escherichia coli sp., Proteus sp.* and *Acinetobacter sp.* are found to be the most sensitive microorganisms to TCS. On the other hand, *Pseudomonas aeruginosa sp.* and *Clostridium difficile sp.* which are generally encountered in hospitals and are difficult to remove are stated as the microorganisms not adversely affected from TCS (APUA, 2011). In this respect, since the environmental conditions (i.e. temperature, sample, the solvent in which TCS stock solution is prepared, used substrate for the microorganisms, etc.) are different for each study, the obtained results and the species detected in the presence of TCS may also become different. Therefore, each study conducted for the determination of microbial composition is unique for the circumstances it is performed and each should be assessed on its own merit.

Based upon the study done for the designation of microbial composition and summarized in the upper paragraphs, it can be concluded that the number of the microbial species was found almost same for acclimated and non-acclimated cultures; it did not change with the case of TCS acclimation of the culture but there could be differences in the species observed with the applied TCS dosage and the acclimation period. Furthermore, it was seen that some species were eliminated from the environment at the end of certain acclimation and some of them survived thanks to the contaminant acclimation. However, since a great number of species was observed to be common in acclimated and non-acclimated cultures, no precise interpretation could be made regarding the assignation of species effective and dominant for TCS removal. This situation was emerging due to the fact that the exact number of microorganisms could not be detected in specie basis; in other words, the exact dominancy of the species could not be

satisfied with these techniques. Additionally, observation of different species as a result of acclimation can be assessed to be the reason of the differency involved in acclimated and non-acclimated cultures in terms of TCS removal efficiency, kinetics and by-product formation as mentioned in the upper sections. Another significant inference of these foundings is that it is not purely possible to gather an exact implication regarding the importance and roles of the microbial species on TCS removal in the scope of composition determination when the microbial species are higher in number in the aerobic systems. Moreover, it may be quite difficult to entirely come up with the finding of the microorganisms affected by TCS at the end of these molecular biological techniques especially if there are certain other biocides present in the environment.

CHAPTER 5

CONCLUSION

Treatment of wastewaters contaminated with the intensively used biocides such as TCS has been the trend topic among the researchers studying in regard to the environmental issues recently. Within the scope of this thesis study, the biological treatability of TCS bearing wastewaters was investigated in detail and it is revealed that significant TCS removal can be achieved in the aerobic activated sludge systems by means of biological degradation thanks to microbial activity, the removal efficiency of the system can be affected from the existing environmental conditions and it can be changed with the certain acclimation to the contaminant and the amount of contaminant in the wastewater of concern.

Another finding of the study is regarding the effect of TCS on ordinary performance of the biological treatment unit. In this respect, this study provides supports to the literature in the subject that the actual level of TCS (i.e. trace amounts) in personal care products and house cleaning products does not pose a threat and create an adverse effect on the normal operation of the biological treatment system. In the study, the activated sludge system was not anticipated to be sorely affected at low TCS concentrations although they are still higher than the utilized products and the amount found in environment while notable inhibition of the actual performance of the system (i.e. COD removal efficiency) was observed to occur only at contaminant concentrations above 10 mg/L for acclimated cultures and less than 1 mg/L for non-acclimated cultures. Additionally, concerning the system COD removal to be negatively affected from the high TCS content of the wastewater but the effect less pronounced with the contaminant

acclimation rather than shock conditions, it is quite obvious that the industries working intermittently and having instantaneous discharges to the treatment plants are the potential threats for the normal operations of the treatment systems and the problems may be encountered in the achievement of the intended discharge standards in effluent wastewaters.

On the other hand, this study indicates that TCS removal is not limited to removal in wastewater and it has to be also monitored in the sewage sludge due to its high hydrophobic nature and tendency to accumulate in solid phases. Hence, the significant amount of TCS was recognized in the acclimated and non-acclimated sludge samples taken for the construction of mass balance and the observed amount in sludge samples after biological treatment increased with the initial TCS amount fed to the reactor. This situation indicates that the adsorption of TCS to sewage sludge should not be ignored in the conditions where the wastewaters with concentrated TCS amount are reaching to the treatment plants and it should be thought as a serious removal mechanism of TCS in the system.

Besides, biodegradation was obtained to be the main mechanism affecting TCS removal in the activated sludge system during the experiments conducted with both acclimated and non-acclimated cultures, excluding the reactor fed with 100 mg/L TCS and operated with non-acclimation culture. For that reactor, sludge adsorption was found to be more profound mechanism instead of biodegration. The mechanism of biodegradation was observed to be enhanced by the adaptation of microbial culture to the contaminant but increasing levels of TCS in the influent wastewater resulted in the inhibition of TCS removal a bit more by the aforementioned mechanism. From this point of view, it can be said that the mechanisms affecting the fate of TCS in the biological treatment system may be varied based upon the prevailing environmental conditions; therefore, the existing conditions should be carefully investigated in order to follow up TCS behavior truely in the system.

Moreover, it was also observed with the study that TCS may transform into different by-products (i.e. 2,4-DCA and 2,4-DCP) during the aerobic biological treatment. The transformation products detected in this study are more polar (i.e. less hydrophobic), less toxic, less persistent and less bioaccumulative than the parent molecule that they can be disregarded in the condition where the focal point is to remove compounds in water posing a risk at lower concentrations in broad time intervals, in other words, compounds with higher chronic toxicity. Still, observation of no TCS in wastewater and sludge after biological treatment should not be considered as its complete removal from the system, possible metabolite formation should be monitored as far as possible and treatment strategy should be planned pondering TCS, these metabolites and their all fates simultaneously in the system.

Regarding the microbial composition, on the other hand, since it was observed numerous common species with acclimated and non-acclimated cultures and there was uncertainty on the dominancy of the species, it was not possible to reach a net and reliable result for the active microbial species and to connect these data with the kinetic findings directly. However, it was understood that the microbial composition is changing depending on the certain adaptation period to TCS and this issue is an important factor for TCS removal efficiency, its kinetics and by-product formation, as well. Moreover, it may be quite difficult to entirely come up with the finding of the microorganisms affected by TCS at the end of these molecular biological techniques especially if there are certain other biocides present in the environment.

CHAPTER 6

RECOMMANDATIONS

As overall recommandations of this thesis study regarding on TCS, following issues are important to consider when the target is to make conclusion on such biocides in environmental systems.

- Scientific studies on the existence, fate and treatment of TCS from the environment should be promoted and appreciated by the governments and private sectors.

- More studies should be made regarding the biodegradability of TCS through the conventional activated sludge systems by considering the water and sludge phase together since sludge adsorption is an important phenomena in these systems and it is a must to take it into consideration in order to get healthy results and obtain true removal efficiencies within the systems.

- Potential transformation by-products should also be monitored within the scope of the researches focused on the fate of TCS.

- During the laboratory experiments, glass labware should be prefered instead of plastic ones to eliminate sorption as well as possible.

- Considering the hydrophobic nature of TCS, extra care should be given to prepare laboratory stock solution in appropriate solvents.

- Since this compound is one of the micropollutants and exist in the environment in trace amounts, devices giving more sensitive measurements such as LC-MS, LC-MS/MS, GC-MS and GC-MS/MS should be used as possible in the experimental studies.

- Potential substitutes for TCS should be investigated for the industrial sectors using it in the production processes of numerous products.

- National inventory of TCS in terms of the amount produced, imported and consumed should be outlined immediately.

- Public awareness should be raised on the adverse effects and potential risks associated with TCS.

REFERENCES

Adolfsson-Erici, M., Pettersson, M., Parkkonen, J. and Sturve, J. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere*. 2002, 46 (9-10), 1485–9.

Agenson, K.O., Oh, J.I. and Urase, T. Retention of a wide variety of organic pollutants by different nanofiltration/reverse osmosis membranes: controlling parameters of process. *Journal of Membrane Sciences*. 2003, 225 (1-2), 91-103.

Allmyr, M. On the Fate of Triclosan in Humans. Stockholm University, Karolinska Institute, Department of Dental Medicine, Stockholm, Sweeden, 2009.

APHA, Standard methods for the examination of water and wastewater, American Public Health Association/American Water Works Association/Water Environment Federation, Washington, D.C, 1998.

APUA, Triclosan, White Paper prepared by The Alliance for the Prudent Use of Antibiotics (APUA), Boston, MA 02111, 2011.

Arnold, W.A., McNeill, K., Packer, J.L., Latch, D.E and Boreen, A.L. Photochemical fate of pharmaceutical compounds discharged and detected in natural waters. Department of Chemistry, University of Minnesota, USA, 2003.

Aufiero, M., Butler, C. and Jaser, J. An analysis of methods, for detecting triclosan and removal of triclosan from water using activated carbon and zeolites. Worcester Polytechnic Institute, Worcester, MA, 2012.

Bester, K. Triclosan in a sewage treatment process—balances and monitoring data. *Water Research*. 2003, 37, 3891–6.

BIOHYPO, Confronting the clinical relevance of biocide induced antibiotic resistance, FP7 Cooperation Work Programme: Food, Agriculture and Fisheries, and Biotechnologies, FP7-KBBE-2008-2B, Acronym BIOHYPO, 2010-2012.

Canard, B., Garnier, T., Lafay, B., Christen, R. and Cole, S.T. Phylogenetic analysis of pathogenic anaerobe clostridium perfringens using the 16SrRNA nucleotide sequence. *Int J Syst Bacteriol*. 1992, 42 (2), 312-4.

Canosa, P., Morales, S.; Rodriguez, I., Rubi, E., Cela, R., and Gomez, M. Aquatic degradation of triclosan and formation of toxic chlorophenols in presence of low concentration of free chlorine. *Anal. Bioanal. Chem*. 2005, 383, 1119-26.

Cha, J. and Cupples, A.M. Detection of the antimicrobials triclocarban and triclosan in agricultural soils following land application of municipal biosolids. *Water Research*. 2009, 43, 2522-30.

Chen, X., Nielsen, J.L., Furgal, K., Liu, Y., Lolas, I.B. and Bester, K. Biodegradation of triclosan and formation of methyl-triclosan in activated sludge under aerobic conditions. *Chemosphere*. 2011, 84 (4), 452-6.

Chen, X., Richard, J., Liu, Z., Dopp, E., Tuerk, J. and Bester, K. Ozonation products of triclosan in advanced wastewater treatment. *Water Research*. 2012, 46 (7), 2247-56.

CIBA, Irgasan, Irgacare-Toxicological and ecological data; Official registration, Brochure 2521; Publication AgB2521e, Ciba Specialty Chemical Holding Inc., 1998.

Ciba-Geigy Limited. Report on vapour pressure curve of triclosan. Unpublished test report, Ciba-Geigy Limited, Basel, Switzerland, 1990d.

Ciba-Geigy Limited. Report on hydrolysis of triclosan as a fundction of pH.. Unpublished test report. Basel, Switzerland, 1990f.

Concha, M. and Shepler, K. Anaerobic aquatic metabolism of [14C]2,4-D Acid. Dow Elanco. Data package ABM-148646-E, DPR#142-155. Department of Pesticide Regulation, Sacramento, CA, 1994.

Dann, A. and Hontela, A. Triclosan: environmental exposure, toxicity and mechanisms of action. *Journal of Applied Toxicology*. 2011, 31, 285-311.

Dilek, F.B., Gökçay, C.F. and Yetiş, U. Combined effects of Ni (II) and Cr (VI) on activated sludge. *Water Research*. 1998, 32, 303–12.

Dolar, D. and Kosutic, K. Removal of pharmaceuticals by ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). *Comprehensive Analytical Chemistry*. 2013, 62, 319-44.

EU SCCP. Scientific Committee on Consumer Safety (SCCS). Opinion on triclosan. Antimicrobial Resistance. 2010, SCCP/1251/09.

Federle, T.W., Kaiser, S.K. and Nuck, B.A. Fate and effects of triclosan in activated sludge. *Environmental Toxicology and Chemistry*. 2002, 21, 1330-7.

Fernandez-Fuentes, M.A., Morente, E.O., Abriouel, H., Pulido, R.P. and Galvez, A. Isolation and identification of bacteria from organic foods: Sensitivity to biocides and antibiotics. *Food Control*. 2012, 26 (1), 73-8.

Ferrer, I., Mezcua, M., Jose Gomez, M., Thurman, M.E., Aguera, A., Hernando, M.D. and Fernandez-Alba, A.R. Liquid chromatography/time-of-flight mass spectrometric analyses for the elucidation of the photodegradation products of triclosan in wastewater samples. *Rapid Communications in Mass Spectrometry*. 2004, 18, 443-50.

Fiss, E.M., Rule, K.L. and Vikesland, P.J. Formation of chloroform and other chlorinated byproducts by chlorination of triclosan-containing antibacterial products. *Environmental Science and Technology*. 2007, 41 (7), 2387-94.

Gatidou, G., Thomaidis, N.S., Stasinakis, A.S. and Lekkas, T.D. Simultaneous determination of the endocrine disrupting compounds nonylphenol, nonylphenol ethoxylates, triclosan and bisphenol A in wastewater and sewage sludge by gas chromatography–mass spectrometry. *Journal of Chromatography A*. 2007, 1138, 32–41.

Gaze, W.H., Abdouslam, N., Hawkey, P.M. and Wellington, E.M.H. Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. *Antimicrob. Agents Chemother*. 2005, 49, 1802–7.

Grady, C.P.L. Jr., Smets, B.F. and Barbeau, D.S. Variability in kinetic parameter estimates: a review of possible causes and a proposed terminology. *Water Research*. 1996, 30, 742–8.

Genç, N. Fermentatif hidrojen üretim prosesinin modellenmesi. *Fen Bilimleri Dergisi*. 2010, 14 (2), 87-97.

Government of Canada. Preliminary Assessment: Triclosan. Environment Canada, Health Canada, 2012.

HACH, Water Analysis Handbook, 2nd ed., Loveland, HACH Company, 1992.

Halden, R.U. and Paull, D.H. Co-occurrence of triclocarban and triclosan in U.S. Water Resources. *Environmental Science and Technology*. 2005, 39 (6), 1420-6.

Hanstveit, A.O. and Hamwijk, C. Environmental Report. V5330/01. Unpublished report, Ciba Specialty Chemicals Inc., 2003.

Heidler, J. and Halden, R.U. Mass balance assessment of triclosan removal during conventional sewage treatment. *Chemosphere*. 2007, 66 (2), 362-9.

Hua, W., Bennett, E.R. and Letcher, R.J. Triclosan in waste and surface waters from the upper Detroit River by liquid chromatography-electrospray-tandem quadrupole mass spectrometry. *Environment International*. 2005, 31, 621–30.

Hyland, K.C., Dickenson, E.R.V., Drewes, J.E. and Higgins, C.P. Sorption of ionized and neutral emerging trace compounds onto activated sludge from different wastewater treatment configurations. *Water Research*. 2012, 46, 1958-68.

Ikehata, K., Naghashkar, N.J. and El-Din, M.G. Degradation of aqueous pharmaceuticals by ozonation and advanced oxidation processes: a review. *Ozone Sci. Eng*. 2006, 28, 353–414.

Ivanov, V. Environmental Microbiology for Engineers. CRC Press, Boca Raton, FL, USA, 2010.

Kanda, R., Griffin, P., James, H.A. and Fothergill, J. Pharmaceutical and personal care products in sewage treatment work. *J Environ Monit*. 2003, 5, 823-30.

Kanetoshi, A., Ogawa, H., Katsura, E. and Kaneshima, H. Chlorination of Irgasan DP300 and formation of dioxins from its chlorinated derivatives. *J. Chromatogr*. 1987, 389, 139–53.

Kim, S.D., Cho, J. Kim, I.S., Vanderford, V.J. and Snyder, S.A. Occurrence and removal of pharmaceuticals and endocrine distruptors in South Korean surface, drinking and wastewaters. *Water Research*. 2007, 41 (5), 1013-21.

Koc, A., Orhon, K.B., Ogutverici, A., Yilmaz, L., Furi, L., Oggioni, M.R., Dilek, F.B. and Yetiş, U. Is adsorption an artifact in experimentation with triclosan? *Desalination and Water Treatment*. 2013, 1-7.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B. and Buxton, H.T. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams 1999-2000: A national reconnaissance. *Environmental Science and Technology*. 2002, 36 (6), 1202-11.

Latch, D., Packer, J., Stender, B., Van Overbeke, J., Arnold, W. and McNeill, K. Aqueous photochemistry of triclosan: Formation of 2,4-dichlorophenol, 2,8-dichlorodibenzo p-dioxin, and oligomerisation products. *Environmental Toxicology and Chemistry*. 2005, 24 (3), 517–25.

Lee, H. and Peart, T.E. Organic contaminants in Canadian municipal sewage sludge. Part I. Toxic or endocrine-disrupting phenolic compounds. *Water Quality Research Journal of Canada*. 2002, 37 (4), 681-96.

Levy, C.W., Roujeinikova, A., Sedelnikova, S., Baker, P.J., Stuitje, A.R., Slabas, A.R., Rice, D.W. and Rafferty, J.B. Molecular Basis of Triclosan Activity. *Nature*.1999, 398 (6726), 383-4.

Lindstrom, A., Buerge, I.J., Poiger, T., Bergqvist, P.A., Muller, M.D. and Buser, H.R. Occurrence and environmental behavior of the bactericide triclosan and ıts methyl derivate in surface waters and in wastewater. *Environmental Science and Technology*. 2002, 36, 2322-9.

Luo, W., Chen, H., Lei, A., Lu, J. and Hu, Z. Estimating cyanobacteria community dynamics and its relationship with environmental factors. *International Journal of Environmental Research and Public Health*. 2014, 11, 1141-60.

Mcavoy, D.C., Schatowitz, B., Jacob, M., Hauk, A. and Eckhoff W.S. Measurement of triclosan in wastewater treatment systems. *Environ Toxicol Chem*. 2002, 21 (7), 1323-9.

McAvoy, D.C., Schatowitz, B., Jacob. M., Hauk A. and Eckhoff W.S. Measurement of triclosan in wastewater treatment systems. *Environmental Toxicol Chem*. 2009, 21, 1323–9.

Mezcua, M., Jose G.M., Ferrer, I., Aguera, A., Dolores, H.M. and Fernandez-Alba, A.R. Evidence of 2,7/2,8-dibenzodichloro-p-dioxin as a photodegradation product of triclosan in water and wastewater samples. *Analytica Chimica Acta*. 2004, 524, 241–7.

Muyzer, G., Hottentrager, S., Teske, A. and Wawer, C. Denaturing gradient gel electrophoresis of PCR amplified 16S rDNA – A new molecular approach to analyse the genetic diversity of mixed microbial communities. In: Akkermans, A.D.L., van Elsas J.D., de Bruijn, F.J. (Eds). Molecular Microbial Ecology Manual, Kluwer Academic Publishing, Dordrecht. 1996, 3.4.4, 1–23.

NICNAS. Priority Existing Chemical Assessment Report No. 30, Triclosan. National Industrial Chemicals Notification and Assessment Scheme (NICNAS), Australian Government, Department of Health and Ageing, Sydney, Australia, 2009.

OECD SIDS. SIDS Initial Assessment Report for 2,4-dichlorophenol. Paris, France. CAS-No. 120-83-2, 2006, SIAM 22.

Ogutverici, A. Triclosan removal by nanofiltration from surface water, (MS Thesis). Middle East Technical University, Faculty of Sciences, Department of Environmental Engineering, 2013.

Oppenlander, T. Photochemical Purification of Water and Air. Weinheim, Germany: Wiley-VCH Verlag, 2003.

Orvos, D.R., Versteeg, D.J., Inauen, J., Capdevielle, M., Rothenstein, A. and Cunningham, V. Aquatic toxicity of triclosan. *Environ. Toxicol. Chem*. 2002, 21, 1338-49.

Pasch, E., Voltmer, L., Gemmell, S., Walter, J. and Walton, K.L.W. Effects of triclosan on the normal intestinal microbiota and on susceptibility to experimental murine colitis. *The Journal of the Federation of American Societies for Experimental Biology*. 2009, 23.

Remberger, M., Sternbeck, J. and Strömberg, K. Screening of triclosan and some brominated phenolic substances in Sweden. Sweedish Environmental Research Institute, Stockholm, Sweeden, 2002.

Roy F. Weston Inc. Bacterial toxicity test on D1063.01. Marks, K.H., Crapo K,C., Ziegenfuss, P.S. and Marks, P.J. (eds). Unpublished Project No. 90-034, Procter & Gamble Company, 1990.

Rule, K.L., Ebbett, V.R. and Vikesland, P.J. Formation of chloroform and chlorinated organics by free-chlorine-mediated oxidation of triclosan. Environmental Science and Technology. 2005, 39, 3176–85.

Sabaliunas, D., Webb, S.F., Hauk, A., Jacob, M. and Eckhoff, W.S. Environmental fate of Triclosan in the River Aire Basin, UK. *Water Research*. 2003, 37 (13), 3145-54.

Samsoe-Petersen, L., Winther-Nielsen, M. and Madsen, T. Fate and effects of triclosan, Project 861, Danish Environmental Protection Agency, Copenhagen, Denmark, 2003.

Silva, A.R.M. and Nogueira, J.M.F. New approach on trace analysis of triclosan in personal care products, biological and environmental matrices. *Talanta*. 2008, 74, 1498–1504.

Silvestre, G., Rodríguez-Abalde, A., Fernandez, B., Flotats, X. and Bonmati A. Biomass adaptation over anaerobic co-digestion of sewage sludge and trapped grease waste. *Bioresource Technology*. 2011, 102 (13), 6830-6.

Singer, H., Müller, S., Tixier, C. and Pillonel, L. Triclosan: Occurrence and fate of a widely used biocide in the environment: Field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environmental Science and Technology*. 2002, 36, 4998-5004.

Somma, M. The analysis of food samples for the presence of genetically, modified organisms. Edited by Querci, M., Jermini, M. and Van den Eede, G. European commission, Joint research centre, 2006.

Springborn Laboratories Inc. Triclosan – Determination of anaerobic aquatic biodegradation. Christensen, K.P. (ed). Unpublished Report No. 93-12-5076, Colgate-Palmolive Company, NJ, USA, 1994a.

Stasinakis, A.S., Mamais, D., Thomaidis, N.S., Danika, E., Gatidou, G. and Lekkas, T.D. Inhibitory effect of triclosan and nonylphenol on respiration rates and ammonia removal in activated sludge systems. *Ecotoxicology and Environmental Safety*. 2008, 70, 199-206.

Stasinakis, A.S., Petalas, A.V., Mamais, D., Thomaidis, N.S., Gatidou, G. and Lekkas, T.D. Investigation of triclosan fate and toxicity in continuous-flow activated sludge systems. *Chemosphere*. 2007, 68, 375-81.

Svensson, A. Ecotoxic substances in sewage sludge – A study of 19 WWTPs in Vastra Gotaland, Sweden. Lansstyrelen I Vastra Gotaland, Report. 2002:39. 2002. In Swedish.

Suarez, S., Dodd, M.D., Omil, F. and Gunten, U. Kinetics of triclosan oxidation by aqueous ozone and consequent loss of antibacterial activity: Relevance to municipal wastewater ozonation. *Water Research*. 2007, 41 (12), 2481–90.

Şahinkaya, E. Modelling aerobic 4-chlorophenol and 2,4-dichlorophenol biodegradatıon-effect of biogenic substrate concentration, (PhD Thesis). Middle East Technical University, Faculty of Sciences, Department of Environmental Engineering, 2006.

Şahinkaya, E. and Dilek, F. B. Biodegradation kinetics of 2,4-dichlorophenol by acclimated mixed cultures. *Journal of Biotechnology*. 2007, 127, 716-26.

Şahinkaya, E. and Dilek, F. B. Effect of biogenic substrate concentration on the performance of sequencing batch reactor treating 4-CP and 2,4-DCP mixtures. *Journal of Hazardous Materials*. 2006, 128, 258-64.

Tchobanoglous, G., Burton, F.L. and Stensel, H.D. Wastewater Engineering: Treatment and Reuse. 4 edn., Metcalf & Eddy Inc., New York, McGraw Hill, 2004.

Thompson, A., Griffin, P., Stuetz, R. and Cartmell E. The fate and removal of triclosan during wastewater treatment. *Water Environ. Res*. 2005, 77, 63.

Tixier, C., Singer H.P., Canonica S. and Muller, R.S. Phototransformation of Triclosan in Surface Waters: A Relevant Elimination Process for This Widely Used Biocides Laboratory Studies, Field Measurements, and Modeling. *Environmental Science and Technology*. 2002, 36, 3482-9.

Republic of Turkey Ministry of Forestry and Water Affairs. Project on the Control of the Pollution Caused by Hazardous Substances-Final Report, 2013.

US EPA. Region/ORD workshop on emerging pollutants-Summary report, 2003 http:// www.epa.gov/osp/regions/emerpoll_rep.pdf

US EPA. Ecotox Database: Aquatic Report; http://cfpub.epa.gov/ecotox/report.cfm

US EPA. Reregistration Eligibility Decision for TCS. Office of Prevention Pesticides and Toxic Substances, 2008.

US EPA. National Center for Environmental Assessment Integrated Risk Information System (IRIS) Database, http://www.epa.gov/iris.

Vaiopoulou, E. and Gikas, P. Effects of chromium on activated sludge and on the performance of wastewater treatment plants: A review. Water Research. 2012, 46 (3), 549-70.

Walsh, S.E, Maillard, J.Y., Russell, A.D., Catrenich, C.E., Charbonneau, D.L. and Bartolo, R.G. Development of bacterial resistance to several biocides and effects on antibiotic susceptibility. *Journal of Hospital Infection*. 2003, 55 (2), 98-107.

Wilson, B.A., Smith, V.H., Denoyelles, Jr F. and Larive, C.K. Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. *Environmental Science and Technology*. 2003, 37, 1713–9.

Wind, T., Werner, U., Jacob, M. and Hauk, A. Environmental concentrations of boron, LAS, EDTA, NTA and Triclosan simulated with GREAT-ER in the River Utter. *Chemosphere*. 2004, 54, 1135-44.

Wu, Q., Zhao, X.H. and Zhao, S.Y. Application of PCR-DGGE in research of bacterial diversity in drinking water. *Biomedical and Environmental Sciences*. 2006, 19, 371-4.

Wu, J.L., Lam, N.P., Martens, D., Kettrup, A. and Cai Z. Triclosan determination in water related to wastewater treatment. *Talanta*. 2007, 72, 1650–4.

Xie, Z., Ebinghaus, R., Floser, G., Caba, A. and Ruck, W. Occurrence and distribution of triclosan in the German Bight (North Sea). *Environmental Pollution.* 2008, 156 (3), 1190-5.

Yavuz, M. Investigation of occurrence and fate of biocides in wastewater treatment plants and surface waters, (MS Thesis). Middle East Technical University, Faculty of Sciences, Department of Environmental Engineering, 2013.

Yetiş, U., Demirer, G.N. and Gökçay, C.F. Effect of chromium(VI) on the biomass yield of activated sludge. *Enzyme and Microbial Technology*. 1999, 25, 48–54.

Ying, G.G. and Kookana, R.S. Triclosan in wastewaters and biosolids from Australian wastewater treatment plants. *Environment International*. 2007, 33 (2), 199–205.

URL 1: http://www.chemicalbook.com/ProductChemicalPropertiesCB8228349_EN.htm, Access date: 07.04.2014

URL 2: http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=TR&language=tr&productNumber=533661&brand=ALDRICH&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Faldrich%2F533661%3Flang%3Den, Access date: 07.04.2014

URL 3: http://wiki.biomine.skelleftea.se/biomine/molecular/index_11.html, Access date: 09.02.2014

URL 4: http://www.norman-network.net/?q=node/81, Access date: 07.04.2014

URL 5: http://www.ilpi.com/msds/ref/riskphrases.html, Access date: 07.04.2014

APPENDIX A

MOLECULAR BIOLOGICAL TECHNIQUES

Polymerase Chain Reaction (PCR)

PCR, commonly used in the recent years for various purposes, is an in vitro technique for the enzymatical amplification of the special DNA region placed between the known two sides of the DNA chain. Thanks to PCR, it becomes possible to amplify the million copies of genes from the single gene within a very short time period as almost in a few hours (Somma, 2006).

PCR depends upon the principle mechanism of DNA replication intracellularly (in vivo). At this stage, double-stranded DNA (dsDNA) turns to single-stranded DNA (ssDNA), duplicated and again bounded, respectively. In other words, PCR is comprised of three main operations. Among of these, the first cycle is the formation of single-stranded DNA following the melting of double-stranded DNA at high temperature, namely, denaturation. Temperature of 93-96°C is required in order to achieve complete and successive denaturation of DNA, which is ultimately to broke down the strong hydrogen bonds and to increase the number of non-paired bases. The second operation is the annealing of two oligonucleotides used as primers to the target DNA which is also known as the operation of "primer match". There is no need for elevated temperatures and nearly 55-65°C temperature is adequate for the accomplishment of this step. The final stage is the addition of nucleotide to the primers with the help of DNA polymerase catalyst in the presence of Mg^{2+} ions and the subsequent

extension of the DNA chain (primer extension). The ideal temperature is 72°C for the primer extension (Somma, 2006).

Aforesaid three cycles of PCR technique are summarized in Figure 27 explicitly.

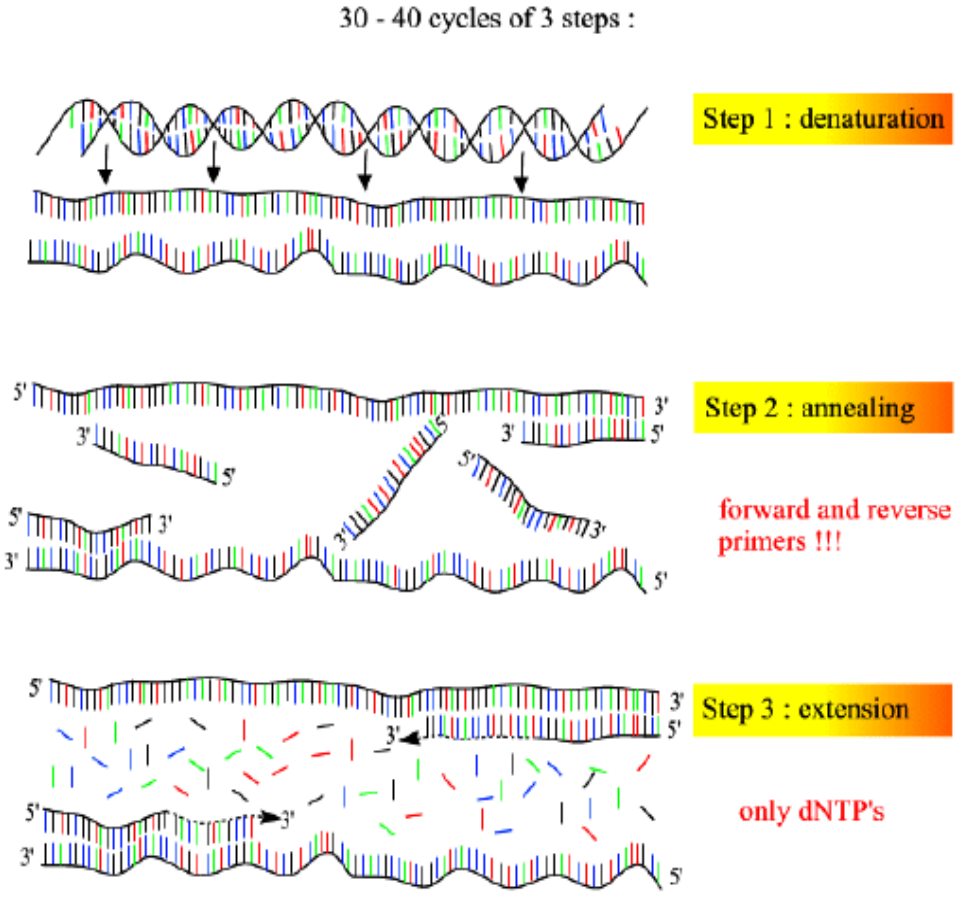


Figure 27 Steps included in PCR amplification process (Somma, 2006)

PCR technique is widely used in the area of molecular biology and molecular medicine for the identification of genes in diagnostics and forensic medicine, and for the cloning of particular DNA fragments. In recent years, this technique has found new usage areas around the world and started to be implemented in the field of bacterial contamination, the presence of genetically modified DNA and the control of the foodstuff ingredients in terms of their authenticity (Somma, 2006).

Denaturating Gradient Gel Electrophoresis (DGGE)

Molecular biological techniques were performed on the sludge samples taken from the pulse-fed sequencing batch reactors at steady state conditions and the variations of the microbial population in time were designated detailly in this stage. For that purpose, bacterial DNA of the sludge samples was extracted, 16SrDNA genes of them were amplified via PCR and the microbial population dynamics were investigated using DGGE. These procedures are schematically represented in Figure 28.

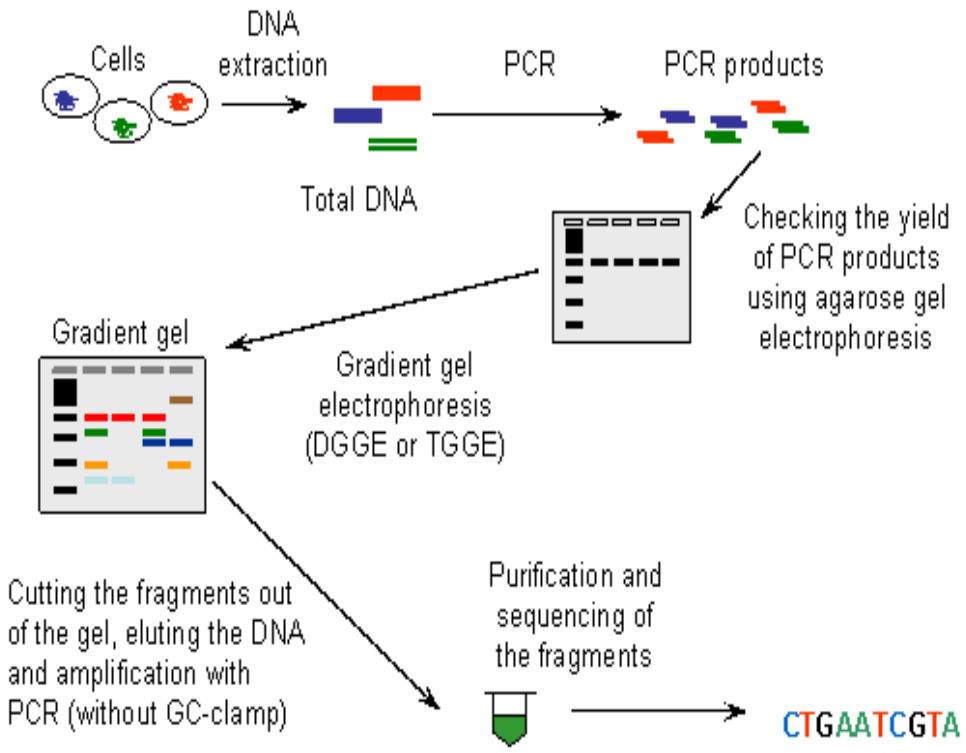


Figure 28 Molecular analysis techniques used for the determination of microbial composition of sludge samples (URL 3)

In the experiments, DNA was extracted with PowerSoil DNA isolation kit (MoBio) according to manufacturer's instructions in order to enhance DNA extraction recovery. Extracted DNA samples were stored at -20ºC. The crude DNA sample was used as a template for PCR. Fragments

corresponding to nucleotide positions 341–926 of the Escherichia coli 16S rRNA gene sequence were amplified with the forward primer GC-BacV3f (5'-CCT ACG GGA GGC AGC AG-3'), to which at the 5' end a GC clamp was added to stabilize the melting behavior of the DNA fragments in the DGGE, and the reverse primer 907r (5'-CCG TCA ATT CMT TTG AGT TT-3') (Muyzer et al., 1996). PCR amplification was performed using Thermocycler T3000 (TECHNE) with the following program: initial denaturation at 95ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 30 s, primer annealing at 50ºC for 1 min and primer extension at 72ºC for 2 min, and final extension at 72ºC for 10 min. The presence of PCR products was confirmed by 1% (w/v) agarose gel electrophoresis and staining with ethidium bromide prior to DGGE analysis. An example of agarose gel electrophoresis is represented in Figure 29.

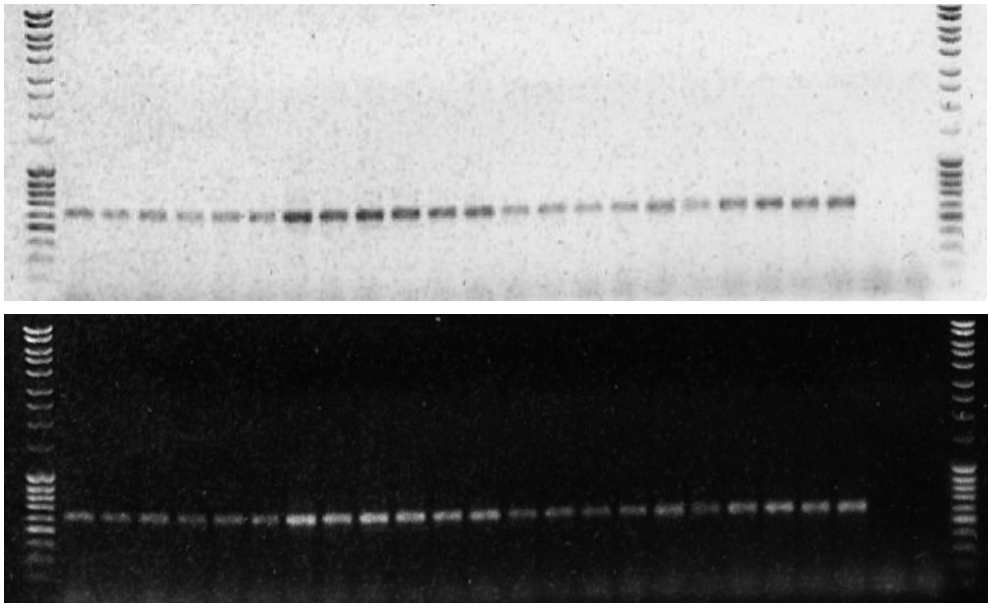


Figure 29 Example of agarose gel electrophoresis conducted for the control of PCR products

DGGE was performed with INGENY phor U-2 apparatus (Ingeny International BV, the Netherlands). PCR samples of 45 mL were loaded onto 8% (w/v) polyacrylamide gel (acrylamide:bisacrylamide stock solution, Sigma-Aldrich) in TAE (40 mM Tris, 20 mM acetic acid, 1 mM

EDTA, pH 8.3) with denaturing gradient ranging from 35 to 60% (100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run at 60ºC with 100V for 22 h. After electrophoresis, the gel was stained in an SYBR Gold solution (100 µL/L in TAE) for 35 min and photographed with 3UV Transilluminator and Digital Camera on Vilber Lourmat Quantum St4 gel documentation system.

Bands in DGGE gels were excised with a razor blade and placed in sterile 200 µl vials. DNA was eluted into 20 µL of water and stored over night at 4ºC. The eluted DNA was used as template in PCR reactions with the primers BacV3f (without GC clamp) and 907r using the same PCR program. After sequence analysis of the purified products, determination of microbial composition was carried out by BLAST (Basic Local Alignment Search Tool) program, nucleotide/protein sequence database that is free and online service from National Center for Biotechnology Information.

APPENDIX B

GENERAL INFORMATION ON BY-PRODUCTS

2,4-DCP

2,4-DCP is a solid substance in the form of white crystals with a molecular weight of 163 g/mole. It has a broad production area in Europe, United States of America, Africa and Asia. It is used as an intermediate in the manufacture of industrial and agricultural products. More commonly, it is known as the intermediate product and major feedstock during the synthesis of the herbicide 2,4-Dichlorophenoxy Acetic Acid. The other usage area as an intermediate includes the formation of preservatives, disinfectants and antiseptics. It is not expected to be exposed directly by humans due to the production process occurring in the closed system and requiring no direct use by humans (OECD SIDS, 2006).

The main mechanisms involved during the environmental fate of 2,4-DCP are photodegradation and volatilization, respectively. Hydrolysis is not considered to be the major mechanisms affecting the fate of this contaminant in the water environment. Additionally, it is also produced as a transformation product of certain other contaminants which should be taken into consideration while studying on its formation mechanism and bioavailability in the environment. It is also reported to have lower tendency to bio-accumulate on the solid surfaces due to its lower BCF values (OECD SIDS, 2006).

2,4 DCP takes place in the hazardous substance list of EPA and it is indicated as the emerging substance in NORMAN List, the list of which is

financially supported by the European Commission and the network of reference laboratories, research centers and related organizations for the monitoring of emerging environmental substances (URL 4). Within the scope of Water Framework Directive (2000/60/EC) , it is also determined as "specific pollutant" in 7 countries, namely, Austria, Belgium, Czech Republic, England, Italy, Romania and Germany (Republic of Turkey Ministry of Forestry and Water Affairs, 2013).

2,4-DCA

2,4-DCA is a colorless liquid with a molecular mass of 177.03 g/mole. It is in the product categories of chlorine compounds and the other known synonym is 2,4-dichloromethoxybenzene. It is mostly used as laboratory chemicals and for the production of other substances. Directive 67/548/EEC does not designate 2,4-DCA as a hazardous substance (URL 1; URL 2). It is also reported as a minor metabolite during the anaerobic degradation of the herbicide 2,4-Dichlorophenoxy Acetic Acid (Concha and Shepler, 1994). The compound is indicated as one of the emerging substances in the category of plant protection products in NORMAN List (URL 4). The physical and chemical properties like water solubility, vapor pressure, octanol-water partition coefficient and volatility are not well known. Therefore, there are not enough data regarding the acute toxicity, bioaccumulation and persistency characteristics of this compound in the literature. Also, there is a serious gap in the literature in terms of its environmental behavior and fate accordingly.

APPENDIX C

RISK PHRASES

Table 17 Description of the risk phrases (URL 5)

Risk Phrase	**Description**
R20/22	Harmful by inhalation and if swallowed.
R21/22	Harmful in contact with skin and if swallowed.
R23	Toxic by inhalation.
R34	Causes burns.
R36	Irritating to eyes.
R37	Irritating to respiratory system.
R38	Irritating to skin.
R36/37/38	Irritating to eyes, respiratory system and skin.
R41	Risk of serious damage to eyes.
R50/53	Very toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment.
R51/53	Toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment.

APPENDIX D

BLAST RESULTS

Table 18 Microbial composition of M1 band

m1

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Comamonas testosteroni CNB-2 chromosome, complete genome	588	1765	100%	1e-165	97%	NC_013446.1
Acidovorax ebreus TPSY chromosome, complete genome	588	1765	100%	1e-165	97%	NC_011992.1
Acidovorax sp. JS42 chromosome, complete genome	588	1765	100%	1e-165	97%	NC_008782.1
Alicycliphilus denitrificans K601 chromosome, complete genome	571	1715	100%	1e-160	96%	NC_015422.1
Alicycliphilus denitrificans BC chromosome, complete genome	571	1715	100%	1e-160	96%	NC_014910.1
Acidovorax sp. KKS102 chromosome, complete genome	560	1681	100%	3e-157	95%	NC_018708.1
Ramlibacter tataouinensis TTB310 chromosome, complete genome	549	549	100%	7e-154	95%	NC_015677.1
Acidovorax avenae subsp. avenae ATCC 19860 chromosome, complete genome	549	1648	100%	7e-154	95%	NC_015138.1
Acidovorax citrulli AAC00-1 chromosome, complete genome	549	1648	100%	7e-154	95%	NC_008752.1
Methylibium petroleiphilum PM1 chromosome, complete genome	532	532	100%	7e-149	94%	NC_008825.1
Delftia sp. Cs1-4 chromosome, complete genome	527	2637	100%	3e-147	94%	NC_015563.1
Rubrivivax gelatinosus IL144, complete genome	521	1565	100%	1e-145	94%	NC_017075.1
Delftia acidovorans SPH-1 chromosome, complete genome	521	2609	100%	1e-145	93%	NC_010002.1
Variovorax paradoxus S110 chromosome 1, complete sequence	510	1021	100%	3e-142	93%	NC_012791.1
Verminephrobacter eiseniae EF01-2 chromosome, complete genome	510	1532	98%	3e-142	93%	NC_008786.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	505	505	100%	1e-140	93%	NZ_CM001438.1
Leptothrix cholodnii SP-6 chromosome, complete genome	499	999	100%	7e-139	92%	NC_010524.1
Rhodoferax ferrireducens T118 chromosome, complete genome	499	999	100%	7e-139	92%	NC_007908.1
Polaromonas naphthalenivorans CJ2 chromosome, complete genome	488	977	100%	2e-135	92%	NC_008781.1
Polaromonas sp. JS666 chromosome, complete genome	483	483	100%	7e-134	91%	NC_007948.1
Azoarcus sp. BH72 chromosome, complete genome	449	1799	100%	7e-124	90%	NC_008702.1
Burkholderia sp. CCGE1001 chromosome 2, complete sequence	444	1332	94%	3e-122	91%	NC_015137.1

Table 19 Microbial composition of M2 band

m2

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Comamonas testosteroni CNB-2 chromosome, complete genome	675	2025	100%	0.0	97%	NC_013446.1
Acidovorax ebreus TPSY chromosome, complete genome	675	2025	100%	0.0	97%	NC_011992.1
Acidovorax sp. JS42 chromosome, complete genome	675	2025	100%	0.0	97%	NC_008782.1
Alicycliphilus denitrificans K601 chromosome, complete genome	658	1975	100%	0.0	96%	NC_015422.1
Alicycliphilus denitrificans BC chromosome, complete genome	658	1975	100%	0.0	96%	NC_014910.1
Acidovorax sp. KKS102 chromosome, complete genome	647	1942	100%	0.0	96%	NC_018708.1
Ramlibacter tataouinensis TTB310 chromosome, complete genome	636	636	100%	6e-180	95%	NC_015677.1
Acidovorax avenae subsp. avenae ATCC 19860 chromosome, complete genome	636	1909	100%	6e-180	95%	NC_015138.1
Acidovorax citrulli AAC00-1 chromosome, complete genome	636	1909	100%	6e-180	95%	NC_008752.1
Methylibium petroleiphilum PM1 chromosome, complete genome	619	619	100%	6e-175	95%	NC_008825.1
Delftia sp. Cs1-4 chromosome, complete genome	614	3071	100%	3e-173	94%	NC_015563.1
Rubrivivax gelatinosus IL144, complete genome	608	1826	100%	1e-171	94%	NC_017075.1
Delftia acidovorans SPH-1 chromosome, complete genome	608	3043	100%	1e-171	94%	NC_010002.1
Variovorax paradoxus S110 chromosome 1, complete sequence	597	1195	100%	3e-168	94%	NC_012791.1
Verminephrobacter eiseniae EF01-2 chromosome, complete genome	597	1792	100%	3e-168	94%	NC_008786.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	592	592	100%	1e-166	93%	NZ_CM001438.1
Leptothrix cholodnii SP-6 chromosome, complete genome	586	1173	100%	6e-165	93%	NC_010524.1
Rhodoferax ferrireducens T118 chromosome, complete genome	586	1173	100%	6e-165	93%	NC_007908.1
Polaromonas naphthalenivorans CJ2 chromosome, complete genome	575	1150	100%	1e-161	93%	NC_008781.1
Polaromonas sp. JS666 chromosome, complete genome	569	569	100%	6e-160	92%	NC_007948.1
Azoarcus sp. BH72 chromosome, complete genome	547	2190	100%	3e-153	91%	NC_008702.1
Burkholderia sp. CCGE1001 chromosome 2, complete sequence	538	1615	98%	2e-150	91%	NC_015137.1

Table 20 Microbial composition of M3 band

m3

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Comamonas testosteroni CNB-2 chromosome, complete genome	680	2042	100%	0.0	97%	NC_013446.1
Acidovorax ebreus TPSY chromosome, complete genome	680	2042	100%	0.0	97%	NC_011992.1
Acidovorax sp. JS42 chromosome, complete genome	680	2042	100%	0.0	97%	NC_008782.1
Alicycliphilus denitrificans K601 chromosome, complete genome	664	1992	100%	0.0	97%	NC_015422.1
Alicycliphilus denitrificans BC chromosome, complete genome	664	1992	100%	0.0	97%	NC_014910.1
Acidovorax sp. KKS102 chromosome, complete genome	652	1958	100%	0.0	96%	NC_018708.1
Ramlibacter tataouinensis TTB310 chromosome, complete genome	641	641	100%	0.0	96%	NC_015677.1
Acidovorax avenae subsp. avenae ATCC 19860 chromosome, complete genome	641	1925	100%	0.0	96%	NC_015138.1
Acidovorax citrulli AAC00-1 chromosome, complete genome	641	1925	100%	0.0	96%	NC_008752.1
Methylibium petroleiphilum PM1 chromosome, complete genome	625	625	100%	1e-176	95%	NC_008825.1
Delftia sp. Cs1-4 chromosome, complete genome	619	3098	100%	6e-175	95%	NC_015563.1
Rubrivivax gelatinosus IL144, complete genome	614	1842	100%	3e-173	94%	NC_017075.1
Delftia acidovorans SPH-1 chromosome, complete genome	614	3071	100%	3e-173	94%	NC_010002.1
Variovorax paradoxus S110 chromosome 1, complete sequence	603	1206	100%	6e-170	94%	NC_012791.1
Verminephrobacter eiseniae EF01-2 chromosome, complete genome	603	1809	100%	6e-170	94%	NC_008786.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	597	597	100%	3e-168	94%	NZ_CM001438.1
Leptothrix cholodnii SP-6 chromosome, complete genome	592	1184	100%	1e-166	93%	NC_010524.1
Rhodoferax ferrireducens T118 chromosome, complete genome	592	1184	100%	1e-166	93%	NC_007908.1
Polaromonas naphthalenivorans CJ2 chromosome, complete genome	580	1161	100%	3e-163	93%	NC_008781.1
Polaromonas sp. JS666 chromosome, complete genome	575	575	100%	1e-161	93%	NC_007948.1
Azoarcus sp. BH72 chromosome, complete genome	542	2168	100%	1e-151	91%	NC_008702.1
Burkholderia sp. CCGE1001 chromosome 2, complete sequence	532	1598	97%	8e-149	91%	NC_015137.1

Table 21 Microbial composition of M4 band

m4

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Aeromonas veronii B565 chromosome, complete genome	492	4923	100%	1e-136	89%	NC_015424.1
Cronobacter turicensis z3032 chromosome, complete genome	486	3396	100%	6e-135	89%	NC_013282.2
Shewanella sp. ANA-3 chromosome 1, complete sequence	486	4347	100%	6e-135	89%	NC_008577.1
Klebsiella variicola At-22 chromosome, complete genome	481	3785	100%	3e-133	88%	NC_013850.1
Pseudomonas fluorescens Pf0-1 chromosome, complete genome	481	2887	100%	3e-133	88%	NC_007492.2
Enterobacter sp. 638, complete genome	481	3352	100%	3e-133	88%	NC_009436.1
Aeromonas salmonicida subsp. salmonicida A449, complete genome	481	4331	100%	3e-133	88%	NC_009348.1
Aeromonas hydrophila subsp. hydrophila ATCC 7966 chromosome, complete genome	481	4812	100%	3e-133	88%	NC_008570.1
Shewanella sp. MR-7 chromosome, complete genome	481	4198	100%	3e-133	88%	NC_008322.1
Shewanella sp. MR-4 chromosome, complete genome	481	4231	100%	3e-133	88%	NC_008321.1
Pseudomonas syrxantha BG33R chromosome, whole genome shotgun sequence	475	2854	100%	1e-131	88%	NZ_CM001514.1
Klebsiella pneumoniae KCTC 2242 chromosome, complete genome	475	3772	100%	1e-131	88%	NC_017540.1
Yersinia pestis A1122 chromosome, complete genome	475	2854	100%	1e-131	88%	NC_017168.1
Klebsiella pneumoniae subsp. pneumoniae HS11286 chromosome, complete genome	475	3800	100%	1e-131	88%	NC_016845.1
Citrobacter rodentium ICC168 chromosome, complete genome	475	3296	100%	1e-131	88%	NC_013716.1
Yersinia pseudotuberculosis PB1/+ chromosome, complete genome	475	3329	100%	1e-131	88%	NC_010634.1
Yersinia pseudotuberculosis IP 31758 chromosome, complete genome	475	3329	100%	1e-131	88%	NC_009708.1
Klebsiella pneumoniae subsp. pneumoniae MGH 78578 chromosome, complete genome	475	3805	100%	1e-131	88%	NC_009648.1
Yersinia pestis Pestoides F chromosome, complete genome	475	3329	100%	1e-131	88%	NC_009381.1
Shewanella amazonensis SB2B chromosome, complete genome	475	3805	100%	1e-131	88%	NC_008700.1
Yersinia pseudotuberculosis IP 32953 chromosome, complete genome	475	3300	100%	1e-131	88%	NC_006155.1
Yersinia pestis CO92 chromosome, complete genome	475	2854	100%	1e-131	88%	NC_003143.1

Table 22 Microbial composition of M5 band

m5

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Comamonas testosteroni CNB-2 chromosome, complete genome	664	1992	100%	0.0	97%	NC_013446.1
Acidovorax ebreus TPSY chromosome, complete genome	664	1992	100%	0.0	97%	NC_011992.1
Acidovorax sp. JS42 chromosome, complete genome	664	1992	100%	0.0	97%	NC_008782.1
Alicycliphilus denitrificans K601 chromosome, complete genome	647	1942	100%	0.0	96%	NC_015422.1
Alicycliphilus denitrificans BC chromosome, complete genome	647	1942	100%	0.0	96%	NC_014910.1
Acidovorax sp. KKS102 chromosome, complete genome	636	1909	100%	6e-180	95%	NC_018708.1
Ramlibacter tataouinensis TTB310 chromosome, complete genome	625	625	100%	1e-176	95%	NC_015677.1
Acidovorax avenae subsp. avenae ATCC 19860 chromosome, complete genome	625	1875	100%	1e-176	95%	NC_015138.1
Acidovorax citrulli AAC00-1 chromosome, complete genome	625	1875	100%	1e-176	95%	NC_008752.1
Methylibium petroleiphilum PM1 chromosome, complete genome	608	608	100%	1e-171	94%	NC_008825.1
Delftia sp. Cs1-4 chromosome, complete genome	603	3015	100%	6e-170	94%	NC_015563.1
Rubrivivax gelatinosus IL144, complete genome	597	1792	100%	3e-168	94%	NC_017075.1
Delftia acidovorans SPH-1 chromosome, complete genome	597	2987	100%	3e-168	94%	NC_010002.1
Variovorax paradoxus S110 chromosome 1, complete sequence	586	1173	100%	6e-165	93%	NC_012791.1
Verminephrobacter eiseniae EF01-2 chromosome, complete genome	586	1759	100%	6e-165	93%	NC_008786.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	580	580	100%	3e-163	93%	NZ_CM001438.1
Leptothrix cholodnii SP-6 chromosome, complete genome	575	1150	100%	1e-161	93%	NC_010524.1
Rhodoferax ferrireducens T118 chromosome, complete genome	575	1150	100%	1e-161	93%	NC_007908.1
Polaromonas naphthalenivorans CJ2 chromosome, complete genome	564	1128	100%	3e-158	92%	NC_008781.1
Polaromonas sp. JS666 chromosome, complete genome	558	558	100%	1e-156	92%	NC_007948.1
Azoarcus sp. BH72 chromosome, complete genome	536	2146	100%	6e-150	91%	NC_008702.1
Burkholderia sp. CCGE1001 chromosome 2, complete sequence	525	1576	100%	1e-146	90%	NC_015137.1

Table 23 Microbial composition of M6 band

m6

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Delftia acidovorans SPH-1 chromosome, complete genome	706	3532	100%	0.0	99%	NC_010002.1
Delftia sp. Cs1-4 chromosome, complete genome	701	3505	100%	0.0	98%	NC_015563.1
Acidovorax sp. KKS102 chromosome, complete genome	667	2003	100%	0.0	97%	NC_018708.1
Acidovorax citrulli AAC00-1 chromosome, complete genome	667	2003	100%	0.0	97%	NC_008752.1
Polaromonas sp. JS666 chromosome, complete genome	667	667	100%	0.0	97%	NC_007948.1
Acidovorax avenae subsp. avenae ATCC 19860 chromosome, complete genome	662	1986	100%	0.0	97%	NC_015138.1
Rhodoferax ferrireducens T118 chromosome, complete genome	656	1313	100%	0.0	96%	NC_007908.1
Alicycliphilus denitrificans K601 chromosome, complete genome	651	1953	100%	0.0	96%	NC_015422.1
Alicycliphilus denitrificans BC chromosome, complete genome	651	1953	100%	0.0	96%	NC_014910.1
Polaromonas naphthalenivorans CJ2 chromosome, complete genome	651	1302	100%	0.0	96%	NC_008781.1
Variovorax paradoxus S110 chromosome 1, complete sequence	640	1280	100%	0.0	96%	NC_012791.1
Acidovorax ebreus TPSY chromosome, complete genome	640	1920	100%	0.0	96%	NC_011992.1
Acidovorax sp. JS42 chromosome, complete genome	640	1920	100%	0.0	96%	NC_008782.1
Ramlibacter tataouinensis TTB310 chromosome, complete genome	612	612	100%	1e-172	94%	NC_015677.1
Verminephrobacter eiseniae EF01-2 chromosome, complete genome	612	1837	100%	1e-172	94%	NC_008786.1
Comamonas testosteroni CNB-2 chromosome, complete genome	601	1803	100%	2e-169	94%	NC_013446.1
Leptothrix cholodnii SP-6 chromosome, complete genome	579	1158	100%	1e-162	93%	NC_010524.1
Rubrivivax gelatinosus IL144, complete genome	568	1704	100%	2e-159	92%	NC_017075.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	556	556	100%	5e-156	92%	NZ_CM001438.1
Methylibium petroleiphilum PM1 chromosome, complete genome	556	556	100%	5e-156	92%	NC_008825.1
Azoarcus sp. BH72 chromosome, complete genome	540	2161	100%	5e-151	91%	NC_008702.1
Burkholderia multivorans ATCC 17616 chromosome 3, complete sequence	531	531	98%	3e-148	91%	NC_010087.1

Table 24 Microbial composition of M7 band

m7

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Shewanella baltica BA175 chromosome, complete genome	691	6895	100%	0.0	98%	NC_017571.1
Shewanella baltica OS223 chromosome, complete genome	691	6901	100%	0.0	98%	NC_011663.1
Shewanella baltica OS185 chromosome, complete genome	691	6801	100%	0.0	98%	NC_009665.1
Shewanella baltica OS155 chromosome, complete genome	691	6851	100%	0.0	98%	NC_009052.1
Shewanella putrefaciens CN-32 chromosome, complete genome	686	5445	100%	0.0	98%	NC_009438.1
Shewanella sp. W3-18-1 chromosome, complete genome	686	5417	100%	0.0	98%	NC_008750.1
Shewanella putrefaciens 200 chromosome, complete genome	680	5423	100%	0.0	97%	NC_017566.1
Shewanella frigidimarina NCIMB 400 chromosome, complete genome	658	5926	100%	0.0	96%	NC_008345.1
Shewanella oneidensis MR-1 chromosome, complete genome	652	5876	100%	0.0	96%	NC_004347.2
Shewanella sp. ANA-3 chromosome 1, complete sequence	652	5827	100%	0.0	96%	NC_008577.1
Shewanella sp. MR-7 chromosome, complete genome	641	5777	100%	0.0	96%	NC_008322.1
Shewanella sp. MR-4 chromosome, complete genome	641	5777	100%	0.0	96%	NC_008321.1
Shewanella denitrificans OS217, complete genome	619	4935	100%	6e-175	95%	NC_007954.1
Shewanella amazonensis SB2B chromosome, complete genome	614	4897	100%	3e-173	94%	NC_008700.1
Shewanella sediminis HAW-EB3 chromosome, complete genome	575	6882	100%	1e-161	93%	NC_009831.1
Shewanella violacea DSS12 chromosome, complete genome	569	7900	100%	6e-160	92%	NC_014012.1
Shewanella woodyi ATCC 51908 chromosome, complete genome	564	5593	100%	3e-158	92%	NC_010506.1
Aeromonas veronii B565 chromosome, complete genome	542	5421	100%	1e-151	91%	NC_015424.1
Pseudoalteromonas sp. SM9913 chromosome I, complete sequence	542	4337	100%	1e-151	91%	NC_014803.1
Tolumonas auensis DSM 9187 chromosome, complete genome	542	4304	100%	1e-151	91%	NC_012691.1
Shewanella piezotolerans WP3 chromosome, complete genome	542	4337	100%	1e-151	91%	NC_011566.1
Shewanella halifaxensis HAW-EB4 chromosome, complete genome	536	5366	100%	6e-150	91%	NC_010334.1

Table 25 Microbial composition of M8 band

m8

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Paenibacillus sp. Y412MC10 chromosome, complete genome	294	2357	74%	4e-77	84%	NC_013406.1
Paenibacillus terrae HPL-003 chromosome, complete genome	289	2558	74%	2e-75	84%	NC_016641.1
Paenibacillus mucilaginosus KNP414 chromosome, complete genome	289	3759	74%	2e-75	84%	NC_015690.1
Paenibacillus polymyxa SC2 chromosome, complete genome	289	3987	74%	2e-75	84%	NC_014622.1
Paenibacillus polymyxa E681 chromosome, complete genome	289	3414	74%	2e-75	84%	NC_014483.1
Bacillus selenitireducens MLS10 chromosome, complete genome	272	1908	74%	2e-70	83%	NC_014219.1
Bacillus cellulosilyticus DSM 2522 chromosome, complete genome	267	2664	74%	9e-69	83%	NC_014829.1
Desulfitobacterium hafniense Y51 chromosome, complete genome	261	1568	74%	4e-67	83%	NC_007907.1
Desulfitobacterium dehalogenans ATCC 51507 chromosome, complete genome	257	1546	75%	5e-66	82%	NC_018017.1
Paenibacillus sp. JDR-2 chromosome, complete genome	257	3093	74%	5e-66	82%	NC_012914.1
Thermobacillus composti KWC4 chromosome, complete genome	255	1268	74%	2e-65	82%	NC_019897.1
Geobacillus sp. WCH70 chromosome, complete genome	252	2522	74%	2e-64	82%	NC_012793.1
Desulfitobacterium dichloroeliminans LMG P-21439 chromosome, complete genome	250	1502	74%	9e-64	82%	NC_019903.1
Brevibacillus brevis NBRC 100599, complete genome	250	3639	74%	9e-64	82%	NC_012491.1
Geobacillus thermoleovorans CCB_US3_UF5 chromosome, complete genome	246	2176	74%	1e-62	82%	NC_016593.1
Geobacillus thermoglucosidasius C56-YS93 chromosome, complete genome	246	2467	74%	1e-62	82%	NC_015660.1
Geobacillus sp. Y4.1MC1 chromosome, complete genome	246	2467	74%	1e-62	82%	NC_014650.1
Geobacillus sp. C56-T3 chromosome, complete genome	246	1929	74%	1e-62	82%	NC_014206.1
Anoxybacillus flavithermus WK1 chromosome, complete genome	246	1949	74%	1e-62	82%	NC_011567.1
Geobacillus thermodenitrificans NG80-2 chromosome, complete genome	246	2400	74%	1e-62	82%	NC_009328.1
Bacillus licheniformis ATCC 14580 chromosome, complete genome	244	1714	74%	4e-62	82%	NC_006270.3
Bacillus licheniformis DSM 13 = ATCC 14580 chromosome, complete genome	244	1714	74%	4e-62	82%	NC_006322.1

Table 26 Microbial composition of M9 band

m9

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Rubrivivax gelatinosus IL144, complete genome	630	1892	100%	3e-178	95%	NC_017075.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	614	614	100%	3e-173	94%	NZ_CM001438.1
Methylibium petroleiphilum PM1 chromosome, complete genome	614	614	100%	3e-173	94%	NC_008825.1
Leptothrix cholodnii SP-6 chromosome, complete genome	592	1184	100%	1e-166	93%	NC_010524.1
Ramlibacter tataouinensis TTB310 chromosome, complete genome	582	582	98%	8e-164	93%	NC_015677.1
Acidovorax sp. KKS102 chromosome, complete genome	577	1731	98%	4e-162	93%	NC_018708.1
Acidovorax avenae subsp. avenae ATCC 19860 chromosome, complete genome	555	1665	98%	2e-155	92%	NC_015138.1
Acidovorax citrulli AAC00-1 chromosome, complete genome	555	1665	98%	2e-155	92%	NC_008752.1
Variovorax paradoxus S110 chromosome 1, complete sequence	549	1099	98%	8e-154	92%	NC_012791.1
Acidovorax ebreus TPSY chromosome, complete genome	549	1648	98%	8e-154	92%	NC_011992.1
Acidovorax sp. JS42 chromosome, complete genome	549	1648	98%	8e-154	92%	NC_008782.1
Polaromonas sp. JS666 chromosome, complete genome	544	544	98%	4e-152	91%	NC_007948.1
Delftia sp. Cs1-4 chromosome, complete genome	538	2692	98%	2e-150	91%	NC_015563.1
Alicycliphilus denitrificans K601 chromosome, complete genome	538	1615	98%	2e-150	91%	NC_015422.1
Alicycliphilus denitrificans BC chromosome, complete genome	538	1615	98%	2e-150	91%	NC_014910.1
Comamonas testosteroni CNB-2 chromosome, complete genome	538	1615	98%	2e-150	91%	NC_013446.1
Rhodoferax ferrireducens T118 chromosome, complete genome	538	1076	98%	2e-150	91%	NC_007908.1
Delftia acidovorans SPH-1 chromosome, complete genome	532	2664	98%	8e-149	91%	NC_010002.1
Herbaspirillum seropedicae SmR1 chromosome, complete genome	531	1593	99%	3e-148	91%	NC_014323.1
Thiomonas sp. 3As, complete genome	527	527	98%	4e-147	91%	NC_014145.1
Thiomonas intermedia K12 chromosome, complete genome	527	527	98%	4e-147	91%	NC_014153.1
Verminephrobacter eiseniae EF01-2 chromosome, complete genome	527	1582	98%	4e-147	91%	NC_008786.1

Table 27 Microbial composition of M10 band

m10

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Janthinobacterium sp. Marseille chromosome, complete genome	257	515	57%	5e-66	87%	NC_009659.1
Thiomonas sp. 3As, complete genome	246	246	99%	1e-62	78%	NC_014145.1
Rubrivivax gelatinosus IL144, complete genome	246	740	80%	1e-62	81%	NC_017075.1
Thiomonas intermedia K12 chromosome, complete genome	246	246	99%	1e-62	78%	NC_014153.1
Herminiimonas arsenicoxydans chromosome, complete genome	246	493	57%	1e-62	86%	NC_009138.1
Herbaspirillum seropedicae SmR1 chromosome, complete genome	244	734	58%	4e-62	86%	NC_014323.1
Methylotenera versatilis 301 chromosome, complete genome	243	729	58%	1e-61	86%	NC_014207.1
Nitrosospira multiformis ATCC 25196 chromosome, complete genome	243	243	58%	1e-61	86%	NC_007614.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	241	241	80%	5e-61	80%	NZ_CM001438.1
Collimonas fungivorans Ter331 chromosome, complete genome	241	723	57%	5e-61	86%	NC_015856.1
Gallionella capsiferriformans ES-2 chromosome, complete genome	239	718	58%	2e-60	85%	NC_014394.1
Candidatus Accumulibacter phosphatis clade IIA str. UW-1 chromosome, complete genome	233	467	58%	9e-59	85%	NC_013194.1
Dechlorosoma suillum PS chromosome, complete genome	231	463	58%	3e-58	85%	NC_016616.1
Leptothrix cholodnii SP-6 chromosome, complete genome	231	463	60%	3e-58	84%	NC_010524.1
Acidovorax sp. KKS102 chromosome, complete genome	230	690	80%	1e-57	80%	NC_018708.1
Burkholderia gladioli BSR3 chromosome 2, complete sequence	230	460	59%	1e-57	84%	NC_015376.1
Burkholderia gladioli BSR3 chromosome 1, complete sequence	230	690	59%	1e-57	84%	NC_015381.1
Methylibium petroleiphilum PM1 chromosome, complete genome	230	230	80%	1e-57	80%	NC_008825.1
Thauera sp. MZ1T chromosome, complete genome	226	905	58%	2e-56	84%	NC_011662.2
Methylobacillus flagellatus KT, complete genome	226	452	58%	2e-56	84%	NC_007947.1
Dechloromonas aromatica RCB, complete genome	226	905	58%	2e-56	84%	NC_007298.1
Burkholderia glumae BGR1 chromosome 1, complete sequence	224	673	59%	5e-56	84%	NC_012724.2

Table 28 Microbial composition of M11 band

m11

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Lacinutrix sp. 5H-3-7-4 chromosome, complete genome	652	1305	100%	0.0	96%	NC_015638.1
Aequorivita sublithincola DSM 14238 chromosome, complete genome	542	1084	100%	1e-151	91%	NC_018013.1
Flavobacterium branchiophilum FL-15, complete genome	536	1609	100%	6e-150	91%	NC_016001.1
Gramella forsetii KT0803 chromosome, complete genome	536	1609	100%	6e-150	91%	NC_008571.1
Cellulophaga lytica DSM 7489 chromosome, complete genome	525	2096	100%	1e-146	90%	NC_015167.1
Flavobacterium indicum GPTSA100-9, complete genome	520	2080	100%	6e-145	90%	NC_017025.1
Zunongwangia profunda SM-A87 chromosome, complete genome	503	1510	100%	6e-140	89%	NC_014041.1
Myroides odoratus DSM 2801 chromosome, whole genome shotgun sequence	497	497	100%	3e-138	89%	NZ_CM001437.1
Flavobacterium johnsoniae UW101 chromosome, complete genome	492	2953	100%	1e-136	89%	NC_009441.1
Nonlabens dokdonensis DSW-6, complete genome	486	973	100%	6e-135	89%	NC_020156.1
Krokinobacter sp. 4H-3-7-5 chromosome, complete genome	486	1454	100%	6e-135	89%	NC_015496.1
Maribacter sp. HTCC2170 chromosome, complete genome	486	973	100%	6e-135	89%	NC_014472.1
Flavobacterium psychrophilum JIP02/86 chromosome, complete genome	486	2920	100%	6e-135	89%	NC_009613.1
Psychroflexus torquis ATCC 700755 chromosome, complete genome	475	1427	100%	1e-131	88%	NC_018721.1
Capnocytophaga canimorsus Cc5 chromosome, complete genome	475	1427	100%	1e-131	88%	NC_015846.1
Flavobacterium columnare ATCC 49512 chromosome, complete genome	459	2295	100%	1e-126	87%	NC_016510.2
Ornithobacterium rhinotracheale DSM 15997 chromosome, complete genome	453	1360	100%	6e-125	87%	NC_018016.1
Zobellia galactanivorans chromosome, complete genome	453	907	100%	6e-125	87%	NC_015844.1
Cellulophaga algicola DSM 14237 chromosome, complete genome	453	2267	100%	6e-125	87%	NC_014934.1
Weeksella virosa DSM 16922 chromosome, complete genome	442	2206	100%	1e-121	87%	NC_015144.1

Table 29 Microbial composition of M13 band

m13

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Pseudoxanthomonas spadix BD-a59 chromosome, complete genome	625	625	100%	1e-176	95%	NC_016147.2
Stenotrophomonas maltophilia K279a chromosome, complete genome	608	2401	100%	1e-171	94%	NC_010943.1
Xanthomonas campestris pv. raphani 756C chromosome, complete genome	597	1195	100%	3e-168	94%	NC_017271.1
Xanthomonas oryzae pv. oryzicola BLS256 chromosome, complete genome	597	1195	100%	3e-168	94%	NC_017267.1
Pseudoxanthomonas suwonensis 11-1 chromosome, complete genome	597	1187	99%	3e-168	94%	NC_014924.1
Xanthomonas albilineans GPE PC73 chromosome, complete genome	597	1195	100%	3e-168	94%	NC_013722.1
Xanthomonas oryzae pv. oryzae KACC 10331 chromosome, complete genome	597	1195	100%	3e-168	94%	NC_006834.1
Xanthomonas axonopodis pv. citri str. 306 chromosome, complete genome	597	1195	100%	3e-168	94%	NC_003919.1
Xanthomonas campestris pv. campestris str. ATCC 33913 chromosome, complete genome	597	1195	100%	3e-168	94%	NC_003902.1
Xanthomonas axonopodis pv. citrumelo F1 chromosome, complete genome	592	1184	100%	1e-166	93%	NC_016010.1
Xanthomonas campestris pv. vesicatoria str. 85-10 chromosome, complete genome	592	1184	100%	1e-166	93%	NC_007508.1
Stenotrophomonas maltophilia JV3 chromosome, complete genome	575	2301	100%	1e-161	93%	NC_015947.1
Xylella fastidiosa M23 chromosome, complete genome	536	1073	100%	6e-150	91%	NC_010577.1
Xylella fastidiosa 9a5c chromosome, complete genome	536	1073	100%	6e-150	91%	NC_002488.3
Frateuria aurantia DSM 6220 chromosome, complete genome	503	2013	99%	6e-140	90%	NC_017033.1
Rhodanobacter sp. 2APBS1, complete genome	497	995	99%	3e-138	89%	NC_020541.1

Table 30 Microbial composition of M14 band

m14

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Flavobacterium indicum GPTSA100-9, complete genome	420	1681	100%	6e-115	86%	NC_017025.1
Flavobacterium columnare ATCC 49512 chromosome, complete genome	414	2073	100%	3e-113	85%	NC_016510.2
Flavobacterium branchiophilum FL-15, complete genome	403	1211	100%	6e-110	85%	NC_016001.1
Ornithobacterium rhinotracheale DSM 15997 chromosome, complete genome	398	1194	100%	3e-108	85%	NC_018016.1
Flavobacterium johnsoniae UW101 chromosome, complete genome	398	2388	100%	3e-108	85%	NC_009441.1
Nonlabens dokdonensis DSW-6, complete genome	381	763	100%	3e-103	84%	NC_020156.1
Flavobacterium psychrophilum JIP02/86 chromosome, complete genome	381	2289	100%	3e-103	84%	NC_009613.1
Myroides odoratus DSM 2801 chromosome, whole genome shotgun sequence	359	359	100%	1e-96	83%	NZ_CM001437.1
Capnocytophaga canimorsus Cc5 chromosome, complete genome	351	1055	81%	2e-94	86%	NC_015846.1
Weeksella virosa DSM 16922 chromosome, complete genome	350	1717	100%	9e-94	86%	NC_015144.1
Zunongwangia profunda SM-A87 chromosome, complete genome	350	1050	98%	9e-94	83%	NC_014041.1
Aequorivita sublithincola DSM 14238 chromosome, complete genome	348	696	100%	3e-93	83%	NC_018013.1
Capnocytophaga ochracea DSM 7271 chromosome, complete genome	340	1363	78%	5e-91	86%	NC_013162.1
Krokinobacter sp. 4H-3-7-5 chromosome, complete genome	333	996	98%	9e-89	82%	NC_015496.1
Lacinutrix sp. 5H-3-7-4 chromosome, complete genome	326	652	100%	1e-86	82%	NC_015638.1
Zobellia galactanivorans chromosome, complete genome	320	641	82%	7e-85	84%	NC_015844.1
Maribacter sp. HTCC2170 chromosome, complete genome	315	630	82%	3e-83	84%	NC_014472.1
Cellulophaga lytica DSM 7489 chromosome, complete genome	307	1230	81%	5e-81	84%	NC_015167.1
Gramella forsetii KT0803 chromosome, complete genome	305	917	98%	2e-80	81%	NC_008571.1
Cellulophaga algicola DSM 14237 chromosome, complete genome	296	1482	81%	1e-77	83%	NC_014934.1
Pedobacter heparinus DSM 2366 chromosome, complete genome	292	878	100%	1e-76	80%	NC_013061.1
Mucilaginibacter paludis DSM 18603 chromosome, whole genome shotgun sequence	287	1149	100%	7e-75	80%	NZ_CM001403.1

Table 31 Microbial composition of M15 band

m15

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Methylotenera mobilis JLW8 chromosome, complete genome	669	1339	100%	0.0	97%	NC_012968.1
Methylotenera versatilis 301 chromosome, complete genome	641	1925	100%	0.0	96%	NC_014207.1
Methylovorus sp. MP688 chromosome, complete genome	580	1161	100%	3e-163	93%	NC_014733.1
Methylovorus glucosetrophus SIP3-4 chromosome, complete genome	580	1161	100%	3e-163	93%	NC_012969.1
Methylobacillus flagellatus KT, complete genome	575	1150	100%	1e-161	93%	NC_007947.1
Dechlorosoma suillum PS chromosome, complete genome	514	1028	100%	3e-143	90%	NC_016616.1
Herminiimonas arsenicoxydans chromosome, complete genome	514	1028	100%	3e-143	90%	NC_009138.1
Gallionella capsiferriformans ES-2 chromosome, complete genome	508	1526	100%	1e-141	90%	NC_014394.1
Candidatus Accumulibacter phosphatis clade IIA str. UW-1 chromosome, complete genome	497	995	100%	3e-138	89%	NC_013194.1
Burkholderia multivorans ATCC 17616 chromosome 3, complete sequence	497	497	99%	3e-138	89%	NC_010087.1
Burkholderia multivorans ATCC 17616 chromosome 1, complete sequence	497	1488	99%	3e-138	89%	NC_010084.1
Janthinobacterium sp. Marseille chromosome, complete genome	497	995	100%	3e-138	89%	NC_009659.1
Burkholderia sp. YI23 chromosome 1, complete sequence	492	1947	99%	1e-136	89%	NC_016589.1
Burkholderia multivorans ATCC 17616 chromosome 2, complete sequence	492	492	99%	1e-136	89%	NC_010086.1
Burkholderia ambifaria AMMD chromosome 3, complete sequence	492	492	99%	1e-136	89%	NC_008392.1
Burkholderia ambifaria AMMD chromosome 2, complete sequence	492	492	99%	1e-136	89%	NC_008391.1
Nitrosospira multiformis ATCC 25196 chromosome, complete genome	492	492	100%	1e-136	89%	NC_007614.1
Burkholderia cepacia GG4 chromosome 1, complete sequence	486	968	99%	6e-135	89%	NC_018513.1
Burkholderia gladioli BSR3 chromosome 2, complete sequence	486	973	99%	6e-135	89%	NC_015376.1
Burkholderia gladioli BSR3 chromosome 1, complete sequence	486	1460	99%	6e-135	89%	NC_015381.1
Sideroxydans lithotrophicus ES-1 chromosome, complete genome	486	973	100%	6e-135	89%	NC_013959.1
Thauera sp. MZ1T chromosome, complete genome	486	1947	100%	6e-135	89%	NC_011662.2
Burkholderia phymatum STM815 chromosome 2, complete sequence	486	973	99%	6e-135	89%	NC_010623.1

Table 32 Microbial composition of M16 band

m16

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Paracoccus denitrificans PD1222 chromosome 2, complete sequence	621	621	99%	2e-175	95%	NC_008687.1
Paracoccus denitrificans PD1222 chromosome 1, complete sequence	621	1243	99%	2e-175	95%	NC_008686.1
Ketogulonigenium vulgarum WSH-001 plasmid 1, complete sequence	610	610	99%	4e-172	94%	NC_017386.1
Ketogulonigenium vulgarum WSH-001 chromosome, complete genome	610	2442	99%	4e-172	94%	NC_017384.1
Ketogulonicigenium vulgare Y25 chromosome, complete genome	610	2442	99%	4e-172	94%	NC_014625.1
Ketogulonicigenium vulgare Y25 plasmid pYP1, complete sequence	610	610	99%	4e-172	94%	NC_014621.1
Rhodobacter capsulatus SB 1003 chromosome, complete genome	599	2397	99%	8e-169	94%	NC_014034.1
Ruegeria pomeroyi DSS-3 chromosome, complete genome	599	1798	99%	8e-169	94%	NC_003911.11
Phaeobacter gallaeciensis 2.10 chromosome, complete genome	588	2353	99%	2e-165	93%	NC_018286.1
Phaeobacter gallaeciensis DSM 17395 chromosome, complete genome	588	2353	99%	2e-165	93%	NC_018290.1
Roseobacter denitrificans OCh 114 chromosome, complete genome	582	582	99%	8e-164	93%	NC_008209.1
Rhodobacter sphaeroides ATCC 17029 chromosome 2, complete sequence	571	1715	99%	2e-160	93%	NC_009050.1
Rhodobacter sphaeroides ATCC 17029 chromosome 1, complete sequence	571	571	99%	2e-160	93%	NC_009049.1
Rhodobacter sphaeroides 2.4.1 chromosome 2, complete sequence	571	1143	99%	2e-160	93%	NC_007494.1
Rhodobacter sphaeroides 2.4.1 chromosome 1, complete sequence	571	571	99%	2e-160	93%	NC_007493.1
Roseobacter litoralis Och 149 chromosome, complete genome	560	560	99%	4e-157	92%	NC_015730.1
Ruegeria sp. TM1040 chromosome, complete genome	555	555	99%	2e-155	92%	NC_008044.1
Ruegeria sp. TM1040 mega plasmid, complete sequence	555	1665	99%	2e-155	92%	NC_008043.1
Ruegeria sp. TM1040 plasmid unnamed, complete sequence	555	555	99%	2e-155	92%	NC_008042.1
Jannaschia sp. CCS1 chromosome, complete genome	538	538	99%	2e-150	91%	NC_007802.1
Dinoroseobacter shibae DFL 12 chromosome, complete genome	534	1069	98%	2e-149	91%	NC_009952.1
Parvibaculum lavamentivorans DS-1 chromosome, complete genome	525	525	98%	1e-146	91%	NC_009719.1

Table 33 Microbial composition of M17 band

m17

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Shewanella putrefaciens 200 chromosome, complete genome	697	5534	100%	0.0	98%	NC_017566.1
Shewanella putrefaciens CN-32 chromosome, complete genome	697	5534	100%	0.0	98%	NC_009438.1
Shewanella sp. W3-18-1 chromosome, complete genome	697	5511	100%	0.0	98%	NC_008750.1
Shewanella sp. MR-7 chromosome, complete genome	686	6076	100%	0.0	98%	NC_008322.1
Shewanella baltica BA175 chromosome, complete genome	680	6784	100%	0.0	97%	NC_017571.1
Shewanella baltica OS223 chromosome, complete genome	680	6790	100%	0.0	97%	NC_011663.1
Shewanella baltica OS185 chromosome, complete genome	680	6723	100%	0.0	97%	NC_009665.1
Shewanella baltica OS155 chromosome, complete genome	680	6762	100%	0.0	97%	NC_009052.1
Shewanella oneidensis MR-1 chromosome, complete genome	675	6076	100%	0.0	97%	NC_004347.2
Shewanella sp. MR-4 chromosome, complete genome	675	6043	100%	0.0	97%	NC_008321.1
Shewanella sp. ANA-3 chromosome 1, complete sequence	669	5993	100%	0.0	97%	NC_009577.1
Shewanella frigidimarina NCIMB 400 chromosome, complete genome	647	5827	100%	0.0	96%	NC_008345.1
Shewanella amazonensis SB2B chromosome, complete genome	625	5002	100%	1e-176	95%	NC_008700.1
Shewanella denitrificans OS217, complete genome	619	4935	100%	6e-175	95%	NC_007954.1
Shewanella sediminis HAW-EB3 chromosome, complete genome	575	6788	100%	1e-161	93%	NC_009831.1
Tolumonas auensis DSM 9187 chromosome, complete genome	564	4509	100%	3e-158	92%	NC_012691.1
Pseudoalteromonas sp. SM9913 chromosome I, complete sequence	558	4470	100%	1e-156	92%	NC_014803.1
Shewanella violacea DSS12 chromosome, complete genome	558	7745	100%	1e-156	92%	NC_014012.1
Shewanella piezotolerans WP3 chromosome, complete genome	553	4426	100%	6e-155	92%	NC_011566.1
Shewanella woodyi ATCC 51908 chromosome, complete genome	553	5532	100%	6e-155	92%	NC_010506.1
Aeromonas veronii B565 chromosome, complete genome	547	5477	100%	3e-153	91%	NC_015424.1
Aeromonas salmonicida subsp. salmonicida A449, complete genome	547	4929	100%	3e-153	91%	NC_009348.1

Table 34 Microbial composition of M18 band

m18

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Clavibacter michiganensis subsp. michiganensis NCPPB 382 chromosome, complete genome	627	1254	99%	4e-177	95%	NC_009480.1
Leifsonia xyli subsp. xyli str. CTCB07 chromosome, complete genome	604	604	99%	2e-170	94%	NC_006087.1
Intrasporangium calvum DSM 43043 chromosome, complete genome	588	1176	99%	2e-165	93%	NC_014830.1
Microbacterium testaceum StLB037, complete genome	579	1158	96%	1e-162	94%	NC_015125.1
Arthrobacter sp. Rue61a chromosome, complete genome	577	3463	99%	4e-162	93%	NC_018531.1
Isoptericola variabilis 225 chromosome, complete genome	577	1731	99%	4e-162	93%	NC_015588.1
Cellulomonas fimi ATCC 484 chromosome, complete genome	577	1154	99%	4e-162	93%	NC_015514.1
Arthrobacter aurescens TC1, complete genome	577	3463	99%	4e-162	93%	NC_008711.1
Beutenbergia cavernae DSM 12333 chromosome, complete genome	573	1147	99%	5e-161	93%	NC_012669.1
Micrococcus luteus NCTC 2665 chromosome, complete genome	571	1143	99%	2e-160	92%	NC_012803.1
Streptomyces cattleya NRRL 8057 = DSM 46488 chromosome, complete genome	566	3397	99%	8e-159	92%	NC_017586.1
Streptomyces cattleya NRRL 8057, complete genome	566	3397	99%	8e-159	92%	NC_016111.1
Arthrobacter phenanthrenivorans Sphe3 chromosome, complete genome	566	2264	99%	8e-159	92%	NC_015145.1
Frankia sp. EuI1c chromosome, complete genome	566	1698	99%	8e-159	92%	NC_014666.1
Arthrobacter sp. FB24, complete sequence	566	2830	99%	8e-159	92%	NC_008541.1
Cellulomonas flavigena DSM 20109 chromosome, complete genome	560	1121	99%	4e-157	92%	NC_014151.1
Arthrobacter chlorophenolicus A6 chromosome, complete genome	556	2784	99%	5e-156	92%	NC_011886.1
Frankia sp. QA3 chromosome, whole genome shotgun sequence	555	555	99%	2e-155	92%	NZ_CM001489.1
Cellvibrio gilvus ATCC 13127 chromosome, complete genome	555	1108	99%	2e-155	92%	NC_015671.1
Kocuria rhizophila DC2201, complete genome	555	1665	99%	2e-155	92%	NC_010617.1
Frankia sp. EAN1pec chromosome, complete genome	555	1665	99%	2e-155	92%	NC_009921.1
Streptomyces coelicolor A3(2) chromosome, complete genome	551	3308	96%	2e-154	92%	NC_003888.3

Table 35 Microbial composition of M19 band

m19

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Candidatus Nitrospira defluvii, complete genome	741	741	100%	0.0	100%	NC_014355.1
Frateuria aurantia DSM 6220 chromosome, complete genome	333	1334	83%	9e-89	85%	NC_017033.1
Caldicellulosiruptor owensensis OL chromosome, complete genome	322	967	77%	2e-85	86%	NC_014657.1
Rhodanobacter sp. 2APBS1, complete genome	316	633	83%	9e-84	84%	NC_020541.1
Caldicellulosiruptor lactoaceticus 6A chromosome, complete genome	316	950	77%	9e-84	85%	NC_015949.1
Terriglobus saanensis SP1PR4 chromosome, complete genome	316	316	83%	9e-84	84%	NC_014963.1
Caldicellulosiruptor kristjanssonii 177R1B chromosome, complete genome	316	950	77%	9e-84	85%	NC_014721.1
Caldicellulosiruptor kronotskyensis 2002 chromosome, complete genome	316	939	77%	9e-84	85%	NC_014720.1
Caldicellulosiruptor obsidiansis OB47 chromosome, complete genome	316	950	77%	9e-84	85%	NC_014392.1
Candidatus Solibacter usitatus Ellin6076 chromosome, complete genome	316	633	83%	9e-84	84%	NC_008536.1
Xanthomonas albilineans GPE PC73 chromosome, complete genome	315	630	84%	3e-83	84%	NC_013722.1
Nitrosococcus watsonii C-113 chromosome, complete genome	313	626	100%	1e-82	81%	NC_014315.1
Nitrosococcus oceani ATCC 19707 chromosome, complete genome	313	626	100%	1e-82	81%	NC_007484.1
Terriglobus roseus DSM 18391 chromosome, complete genome	311	622	83%	4e-82	84%	NC_018014.1
Granulicella mallensis MP5ACTX8 chromosome, complete genome	311	311	83%	4e-82	84%	NC_016631.1
Caldicellulosiruptor hydrothermalis 108 chromosome, complete genome	311	934	77%	4e-82	85%	NC_014652.1
Caldicellulosiruptor bescii DSM 6725 chromosome, complete genome	311	934	77%	4e-82	85%	NC_012034.1
Hippea maritima DSM 10411 chromosome, complete genome	309	619	100%	1e-81	81%	NC_015318.1
Conexibacter woesei DSM 14684 chromosome, complete genome	307	307	99%	5e-81	81%	NC_013739.1
Leptospirillum ferriphilum ML-04 chromosome, complete genome	305	611	83%	2e-80	83%	NC_018649.1
Acidobacterium capsulatum ATCC 51196 chromosome, complete genome	305	305	83%	2e-80	83%	NC_012483.1
Geobacter sulfurreducens PCA chromosome, complete genome	303	607	99%	7e-80	81%	NC_002939.5

Table 36 Microbial composition of M20 band

m20

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Comamonas testosteroni CNB-2 chromosome, complete genome	641	1925	100%	0.0	96%	NC_013446.1
Acidovorax ebreus TPSY chromosome, complete genome	636	1909	100%	6e-180	95%	NC_011992.1
Acidovorax sp. JS42 chromosome, complete genome	636	1909	100%	6e-180	95%	NC_008782.1
Acidovorax sp. KKS102 chromosome, complete genome	619	1859	100%	6e-175	95%	NC_018708.1
Alicycliphilus denitrificans K601 chromosome, complete genome	619	1859	100%	6e-175	95%	NC_015422.1
Alicycliphilus denitrificans BC chromosome, complete genome	619	1859	100%	6e-175	95%	NC_014910.1
Ramlibacter tataouinensis TTB310 chromosome, complete genome	608	608	100%	1e-171	94%	NC_015677.1
Acidovorax avenae subsp. avenae ATCC 19860 chromosome, complete genome	608	1826	100%	1e-171	94%	NC_015138.1
Methylibium petroleiphilum PM1 chromosome, complete genome	608	608	100%	1e-171	94%	NC_008825.1
Acidovorax citrulli AAC00-1 chromosome, complete genome	608	1826	100%	1e-171	94%	NC_008752.1
Delftia sp. Cs1-4 chromosome, complete genome	603	3015	100%	6e-170	94%	NC_015563.1
Rubrivivax gelatinosus IL144, complete genome	597	1792	100%	3e-168	94%	NC_017075.1
Delftia acidovorans SPH-1 chromosome, complete genome	597	2987	100%	3e-168	94%	NC_010002.1
Leptothrix cholodnii SP-6 chromosome, complete genome	586	1173	100%	6e-165	93%	NC_010524.1
Rhodoferax ferrireducens T118 chromosome, complete genome	586	1173	100%	6e-165	93%	NC_007908.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	580	580	100%	3e-163	93%	NZ_CM001438.1
Variovorax paradoxus S110 chromosome 1, complete sequence	580	1161	100%	3e-163	93%	NC_012791.1
Verminephrobacter eiseniae EF01-2 chromosome, complete genome	575	1726	100%	1e-161	93%	NC_008786.1
Polaromonas naphthalenivorans CJ2 chromosome, complete genome	569	1139	100%	6e-160	92%	NC_008781.1
Polaromonas sp. JS666 chromosome, complete genome	569	569	100%	6e-160	92%	NC_007948.1
Azoarcus sp. BH72 chromosome, complete genome	531	2124	100%	3e-148	91%	NC_008702.1
Burkholderia sp. CCGE1001 chromosome 2, complete sequence	525	1576	100%	1e-146	90%	NC_015137.1

Table 37 Microbial composition of M21 band

m21

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Comamonas testosteroni CNB-2 chromosome, complete genome	630	1892	98%	3e-178	95%	NC_013446.1
Acidovorax ebreus TPSY chromosome, complete genome	630	1892	98%	3e-178	95%	NC_011992.1
Acidovorax sp. JS42 chromosome, complete genome	630	1892	98%	3e-178	95%	NC_008782.1
Alicycliphilus denitrificans K601 chromosome, complete genome	614	1842	98%	3e-173	95%	NC_015422.1
Alicycliphilus denitrificans BC chromosome, complete genome	614	1842	98%	3e-173	95%	NC_014910.1
Acidovorax sp. KKS102 chromosome, complete genome	603	1809	98%	6e-170	94%	NC_018708.1
Ramlibacter tataouinensis TTB310 chromosome, complete genome	592	592	98%	1e-166	94%	NC_015677.1
Acidovorax avenae subsp. avenae ATCC 19860 chromosome, complete genome	592	1776	98%	1e-166	94%	NC_015138.1
Acidovorax citrulli AAC00-1 chromosome, complete genome	592	1776	98%	1e-166	94%	NC_008752.1
Methylibium petroleiphilum PM1 chromosome, complete genome	586	586	100%	6e-165	93%	NC_008825.1
Delftia sp. Cs1-4 chromosome, complete genome	580	2904	98%	3e-163	93%	NC_015563.1
Rubrivivax gelatinosus IL144, complete genome	575	1726	100%	1e-161	93%	NC_017075.1
Delftia acidovorans SPH-1 chromosome, complete genome	575	2877	98%	1e-161	93%	NC_010002.1
Leptothrix cholodnii SP-6 chromosome, complete genome	564	1128	100%	3e-158	92%	NC_010524.1
Burkholderiales bacterium JOSHI_001 chromosome, whole genome shotgun sequence	558	558	100%	1e-156	92%	NZ_CM001438.1
Variovorax paradoxus S110 chromosome 1, complete sequence	553	1106	98%	6e-155	92%	NC_012791.1
Verminephrobacter eiseniae EF01-2 chromosome, complete genome	553	1659	98%	6e-155	92%	NC_008786.1
Rhodoferax ferrireducens T118 chromosome, complete genome	553	1106	98%	6e-155	92%	NC_007908.1
Polaromonas naphthalenivorans CJ2 chromosome, complete genome	542	1084	97%	1e-151	92%	NC_008781.1
Polaromonas sp. JS666 chromosome, complete genome	536	536	97%	6e-150	91%	NC_007948.1
Azoarcus sp. BH72 chromosome, complete genome	508	2035	100%	1e-141	90%	NC_008702.1
Burkholderia sp. CCGE1001 chromosome 2, complete sequence	503	1510	100%	6e-140	89%	NC_015137.1