The Effect of Inherent Dissolved Organic Carbon on Biodegradation of 4-Nitrophenol in Enhanced Biodegradation Test

K.U. Emerson[a], C.O. Nabena[a], G. Obiechefu[b], U.C. Egbufor[b], E.S. Bejor[c]
[a]University of Newcastle upon Tyne-UK, [b]Imo State Polytechnic Owerri-Nigeria, [c]University of Sheffield-UK
(Email: kemerson@hotmail.co.uk)

Abstract—A ready biodegradability test (RBT) was conducted on 4-nitrophenol (4-NP) using inocula from activated sludge samples of five wastewater treatment plants (WWTPs): Tudhoe Mill, Howden, Cramlington, Sedgeletch, and Washington located in Northeast of England. The tests were performed in line with Organization for Economic Cooperation and Development (OECD) Standardized Tests for Various RBT Criteria. 4-NP was observed to be biodegraded by activated sludge from all WWTPs. There were variations in biological activity across the test samples as measured by the amount of carbon dioxide (CO_2) produced by the respective media. Test samples from Howden and Washington recorded the highest biological activity with 66.81 mg C/l and 69.34 mg C/l of CO_2 respectively. CO_2 of 40mg/l and above was observed to result in decrease of microbial population, except for the sample from Howden Plant, where 66.81mg/l of CO_2 did not affect the microbial population-a condition that could indicate the tolerance of the microbial culture to a fairly toxic environment as seen from the high COD of effluent from Howden WWTP. However, no significant difference (p=0.89) was observed in biomass density of sludge samples from the five WWTPs before and after the biodegradation process.

Keywords—4-nitrophenol, cell density, COD, dissolved organic carbon, inocula, ready biodegradability test

1 Introduction

Biodegradation means the breakdown or decay of materials which takes place when microorganisms utilize organic substance as a source of carbon and energy. Determining biodegradation rate of substances is an important step towards chemical risk assessment. The main substrates utilized in biodegradation constitute microbes (which can be used as a source of nitrogen, carbon, sulphur, phosphorous and other elements required by cells for multiplying) and artificial chemicals (including many organic pollutants) which can represent original sources of energy and carbon for a certain inhabitants The prolonged use of hydrocarbons has been observed to cause pollution in most parts of the environment [15]; and the application of large numbers of microorganisms in biodegradation was said to be the primary process behind the natural attenuation of these hydrocarbons from natural ecosystems [3].

Biodegradation constitutes one of the primary mechanisms widely applied in the elimination of 4-nitrophenol (4-NP) from the natural ecosystems [9]. Although phenols constitute an essential substrate for the selective activity of some microorganisms, their mere presence in wastewater treatment plants has recently become a major concern [20]. This is due to the fact that they are toxic to individuals in the microbial community and subsequently impact on biodegradation rates [22], [2]. The biodegradability of 4-NP depends mainly on the physical and chemical properties of the medium and the presence of 4-NP degraders in the local microbial community [23].

Biodegradability tests are designed to investigate if a chemical is biodegradable, whether partially or completely. With advancements in molecular biology, most biodegradability tests are now conducted in line with Organization for Economic Cooperation and Development (OECD) test guidelines, and at fixed temperatures of 20-25°C. Different Ready Biodegradability Tests (RBTs) formats have been developed, and the general rule is to study how a chemical degrades over a particular period (normally 28 days), after vaccination with a natural bacterial source (inocula). The inocula can be derived from the soil, surface waters, activated sludge, or a combination of these sources (Martin et al, 2011). The source should be non-industrial and must not be pre-exposed to the test chemicals. Positive reference compounds (including sodium acetate, sodium benzoate and aniline) are run together with the test compounds so as to authenticate the test methods [17]. However, the most uncomplicated of these tests is the closed bottle test (OECD, 301D) whereby the test chemical are diluted in an inoculated media with a standard inoculum and mineral medium in a closed glass bottled in the dark for 28 days, with the measurement of BOD over the particular period. In RBTs, degradation is generally measured at regular intervals using parameters like; CO_2 production, oxygen uptake or dissolved organic carbon (DOC). The 'pass' levels for complete biodegradability are 60% removal of BOD or CO_2 evolution or 70% for the DOC removal over 10 day window in 28 days period [14]. This research aims to determine the effect of inherent dissolved organic carbon on the degradation of 4-NP in enhanced biodegradation test. The objectives of the research are (1) to determine the physical and chemical characteristics of activated sludge samples from five wastewater treatment plants in the Northumbrian region of England, (2) to estimate the effect of the physico-chemical parameters on the microbial community sizes in the wastewater treatment plants and on the degradation of 4-NPand (3) to determine the effect of dissolved organic carbon (DOC) on enhanced biodegradability test.

$$= \frac{Mean\ number\ of\ cells\ per\ FOV\ X\ total\ area\ of\ filter\ (mm\,2)}{Area\ of\ FOV\ (mm\,2) X\ volume\ of\ sample\ X\ dilutio} (2.1)$$

(Where: Total area of filter = 133mm²; Area of FOV= 0.01mm²)

2 MATERIALS AND METHODS

2.1 Activated sludge sample collection

The samples were collected from activated sludge chambers of five wastewater treatment plants (WWTPs) in Northumbrian Water Region of Northeast England, using sterile plastic containers (thoroughly washed with Virkon), and stored at 4°C prior to their analysis. The WWTPs are Tudhoe Mill, Howden, Cramlington, Sedgeletch, and Washington.

2.2 Determination of inocula (activated sludge) characteristics

The samples were analyzed for pH using a JENWAY 3310 pH meter. The pH probe was calibrated with a standard buffer prior to use, and the pH determined following Standard Methods for the Examination of Water and Wastewater, APHA (1989). Mixed Liquor Suspended Solid (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) were determined following Standard Methods for the Examination of Water and Wastewater, APHA (1989, part 240-E); while Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) where determined following Standard Methods for the Examination of Water and Wastewater, APHA (1989, part 5220-C & 5210B) respectively. Ammoniacal Nitrogen and Total Kjeldahl Nitrogen (TKN) were analyzed following the Standard Methods for the Examination of Water and Wastewater, APHA (1989, 4500-NH_3 C & 4500 – Norg B).

2.3 Microbial population determination (total bacterial count)

Using 4, 6 diamidino-2-phenylindole (DAPI), the total number of cells/ml in each sample was determined. The DAPI stain was prepared from a 1/100 dilution of one of the samples in phosphate buffer saline (PBS; i.e. 10 µl sample in 1000 µl total volume). 100 µl of each activated sludge samples was mixed with 100 µl DAPI and 800 µl of MilliQ water so as to obtain 1/10 dilutions. These dilutions were then mixed and incubated at room temperature for 15 minutes. The DAPI stained samples were later filtered through Nucleopore black polycarbonate filters (13 mm) held on a sterile Millipore stainless steel vacuum filter unit as follows; for each of the DAPI stained samples, 30 µl was filtered together with 70 µl of MilliQ water. A clean glass slide with a small drop of citifluor was used to air-dry each filter paper (containing the samples) for a few minutes prior to a final addition of an extra drop of citifluor (on the filter). Each slide was then covered with a cover-slip and viewed. An Olympus BX41 Epifluorescence microscope at magnification of x 100 and U-RFL-T- 200 power supply for 100W High Pressure Mercury Burner was used to view the samples; where cells were counted in 20 fields of view (FOV) within the square ocular. The number of cells/ml was calculated using the formula:

Total number of cells per ml

2.4 Total carbon (TC) on samples

The Total Carbon (TC) of each sample was measured using a Leco CS 244 Analyzer. For TC analysis, approximately 0.1 g of powdered sample was weighed and put into a crucible [18], [12]. It was introduced to a combustion furnace with each sample combusted at a high temperature (approximately 1350°C) in a pure oxygen atmosphere to ensure combustion of all carbon forms. The carbon was oxidized to CO_2 which was swept along with oxygen to the analyzer and detected by a thermal conductivity detector.

2.5 Mineral medium preparation

Analytical grade reagents (8.5 g of KH_2PO4, 21.75 g of K_2HPO4, 33.4 g of $Na_2HPO4.2H_2O$ and 0.5 g of NH_4Cl) were used to prepare stock solution A. 27.5 g of $CaCl_2$ was used to prepare stock solution B. 22.5 g of $MgSO_4$.7H20 was used too to prepare stock solution C, and 0.25 g of $FeCl_3.6H_2O$ was also used to prepare stock solution D. All stock solutions were dissolved in sterile distilled water and made up to 1 litre each. In preparing the mineral medium, 1 ml of solutions A, B, C and D was mixed with 800 ml sterile distilled water and made up to 1 litre. 20 ml of the mineral medium was added to 20 ml of the inocula and was used as the control experiment.

2.6 Chemical preparation

4-nitrophenol is the sole carbon source at a concentration of 10 mg C litre-1, and the mineral medium was prepared and sterilized prior to the addition of test chemical (which was filter sterilized using a syringe filter). The test chemical medium is expressed in L^{-1}.

2.7 Inocula preparation

The activated Sludge samples used in the Microcosm experiments were sampled on a single occasion from different wastewater treatment plants, and inoculated into all tests serum bottles at different occasions. This implies that the samples at the same day of inoculation were standardized across all chemicals and that the results allowed for comparisons to be made solely on substrate chemistry.

2.8 Ready biodegradability-CO_2 in sealed vessels (Headspace test)

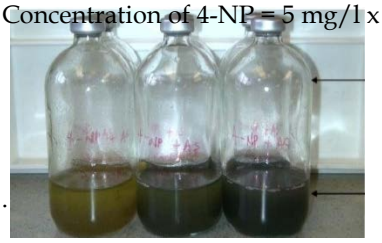
The test for biodegradability were carried out with reference to defined procedures as laid down in OECD 310 (OECD 310, 2006) and ISO 14593 (ISO Standard 14594, 1999). The 125 ml serum bottles containing the test solutions (of 40 ml volume) were sealed with a Teflon rubber septa and aluminium caps, and were used on the test vessels before incubation. Therefore, headspace-to-liquid volume ratio of 2:1 was attained. In the experiment, 1 g C/L stock solution was prepared by dissolving 1.93 g of 4-NP in 1 L of sterile water. A test chemical medium was prepared using 10 ml of the stock solution and making it up to 1 L with mineral medium. 20 ml of the medium was added to 20 ml activated sludge sample (108 CFU/ml) to

make up a total test solution of 40 mg C/L (4-NP, mineral salts medium and inocula) shown in Figure 2.1. The concentration of 4-NP in test samples was determined thus:

Concentration of stock x volume = Concentration in solution x new volume

Concentration in solution = 5 mg C/L

Concentration of 4-NP = 5 mg/l x 1.93 = 9.65 mg/l



Headspace (CO_2 produced)

Test inoculum solution

Fig. 2.1. Sample of test solutions of WWTP activated sludge samples during biodegradation.

The test bottles, in duplicates, were incubated in the dark (using paper foils) at 20°C, in an incubator that has a shaker. The experiments were performed also at intervals, after keeping only 20 ml of the inocula in a sealed vessel and in a 20°C incubator, the chemical test solution was then added at different days (1, 2, 5, and 12 days). The Control for each sample was obtained by mixing the mineral medium with the particular inocula. Biodegradation was observed for a period of 12 days and CO_2 production was determined in the test bottles using a Fisons 8060 Gas Chromatography – Mass Spectroscopy. Total cell count and total carbon were also calculated after biodegradation.

2.9 Incubation

The serum bottles were placed in a IKA® KS 4000 *i* incubator at a temperature of 20°C in the absence of light for a period of 28 days. This was to assess the effect of temperature on the community structure of the activate sludge organisms.

3 Biodegradability assay

Chemical degradation was determined by monitoring the production of CO_2 over time in the microcosms (with two duplicates per treatment). CO_2cumulation was measured in the microcosm headspace, and analyzed using Fisons MD800 (Triess) GC-MS. A headspace sample (100 µl) was injected in split mode and the GC programme and MS data acquisition commenced. Separation was performed on an HP-PLOT-Q capillary column (30m x 0.32mm i.d) packed with 20 µm Q phase. The GC was held isothermally at 35°C with Helium as the carrier gas. The chromatogram of the gas (CO_2) was integrated and quantified. The acquired data were used to ensure that the CO_2 responses were basically produced by living the microbes.

4 Results and discussions

4.1 Chemical characteristics of activated sludge from WWPs

Table 4.1 shows the chemical parameters of activated sludge samples from all the five WWPT. The pH values of the activated sludge are neutral and within the anticipated range [16]. Howden has the highest percentage of solids in activated sludge (94% MLVSS in MLSS) while Tudhoe Mill recorded the least (77%). TKN, NH_3, and Org. N vary across the treatment plants- an indication of their various effluent characteristics. BOD and COD also vary across the different plants, with Howden showing the highest BOD of 3300 mg/l and COD of 6500 mg/l, while Tudhoe Mill has the least BOD of 579 mg/l and COD of 1200 mg/l respectively.

Table 4.1. Chemical parameters of activated sludge samples from five WWTPs before biodegradation test

Parameter	Tudhoe Mill	Howden	Cramlington	Sedgeletch	Washington
pH	6.74	7.11	7.22	7.01	6.92
MLSS (mg/l)	1916.67	5513.33	1966.67	2573.33	2813.33
MLVSS (mg/l)	1476.67	4773.33	1850.00	2076.67	2593.33
NH_3 (mg/l)	11.00	35.00	49.00	96.00	39.00
TKN (mg/l)	140.00	311.00	151.00	172.00	119.00
Org.-N (mg/l)	129.00	276.00	102.00	76.00	80.00
BOD (mg/l)	579.00	3300.00	940.00	2500.00	650.00
COD (mg/l)	1200.00	6500.00	3000.00	4800.00	2000.00
%Volatile Solids	77.00	94.00	87.00	81.00	92.00

4.2 Effect of 4-NP on biomass concentration of activated sludge samples

Figure 4.1 shows biomass concentration of Test samples and Control before and after biodegradation. Tudhoe Mill showed the highest percentage increase of 122% in biomass followed by Howden which showed 106% increase, while Washington and Sedledgech showed 76% and 54% decrease in cell densities respectively. The Control samples for Tudhoe Mill and Howden also recorded 52% and 41% increase respectively in cell densities, while such was not observed for the other WWTPs. Statistical analysis of the initial and final biomass concentrations from all treatment plants (Table 4.2) shows a *p* value of 0.89 which is greater than the alpha level of 0.05. Hence, there was no significant difference in the biomass concentration of all treatment plants.

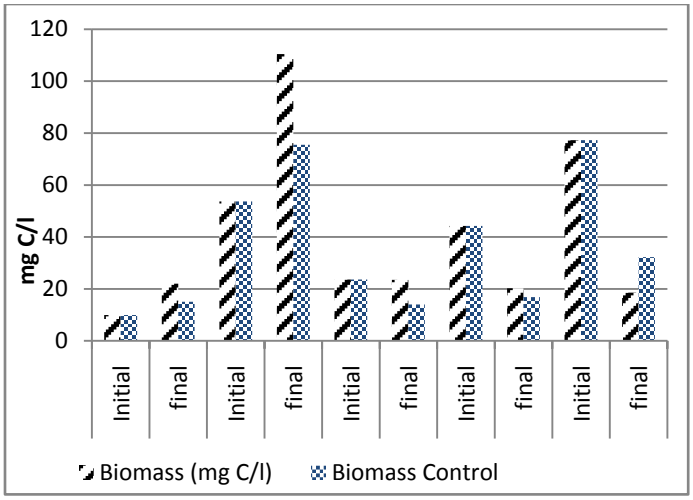


Fig. 4.1. Biomass concentration of test samples and control before and after biodegradation

Table 4.2. ANOVA result of initial and final biomass concentrations from all WWTPs

Source	SS	df	MS	F	P
Treatment [between groups]	19.6841	1	19.6841	0.02	0.891033
Error	9155.2088	8	1144.4011		
SS/Bl					
Total	9174.8929	9			

The increase in biomass concentration of activated sludge samples from Howden and Tudhoe Mill could be attributed to the high concentration of their COD in proportion to their MLSS. Activated sludge from Howden and Tudhoe Mill also contained the highest amount of organic nitrogen, as compared to the other WWTPs. Hence, Howden and Tudhoe Mill Plants received relatively strong effluent which has exposed the degrading microorganisms to relatively high toxicity. The degrading microorganisms from these plants have, thus, become somewhat acclimatized to strong effluents, and can utilize same as carbon source. This implies that these Plants can biodegrade fairly strong effluent with relative ease. This agrees with the findings that although phenols can be toxic to individuals in microbial community [22], they can constitute essential substrate for the selective activity of some microorganisms which have become somewhat acclimatized to toxic condition [20].

On the contrary, treatment plants which showed a decrease in cell densities following biodegradation of 4-NP could be as a result of the test chemical being toxic to the native microorganisms. The decrease could also be attributed to the age of the sludge.

4.3 Effect of inherent organic carbon on biodegradation

Table 4.3 shows the organic carbon, CO_2, and biomass concentrations of samples. The inherent dissolved organic carbon in the sludge solutions at the start of the experiment varied in the order Cramlington >Howden> Washington >Tudhoe mill >Sedgeletech. On the other hand, the extent of biodegradation as measured by the net CO_2 at the end of the experiment varied in the order of Washington >Howden>Sedgeletech> Cramlington>Tudhoe mill. It is also observed that there is increase in net dissolved organic carbon for Washington (20%) and Howden (41%) whereas Cramlington, Tudhoe mill and Sedgeletech show net decreases of 32%, 24% and 15% in dissolved organic carbon.

Table 4.3. Selected chemical parameters of Test samples before and after biodegradation

		Parameters		
WWTP		TOC (mg C/l)	CO_2 (mg C/l)	Biomass (mg C/l)
Tudhoe Mill	Initial	17.13	3.02	9.90
	final	13.00	34.01	21.99
Howden	Initial	19.99	4.03	53.68
	final	28.13	66.81	110.42
Cramlington	Initial	21.40	2.16	23.59
	final	14.60	41.28	23.46
Sedledgech	Initial	15.49	0.48	44.25
	final	13.14	41.25	20.21
Washington	Initial	18.15	0.45	77.24
	final	21.83	69.34	18.55

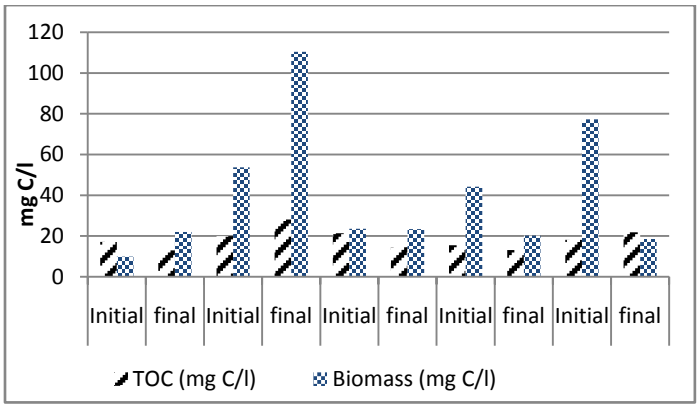


Fig. 4.2. TOC and biomass of test samples at beginning and end of biodegradation test

This result indicates that for the wastewater treatment plants where the net dissolved organic carbon increased, the micro-organisms might have utilized part or whole of the chemical as a source of carbon and energy. Alternatively, they might have used their own carbon as the sole source of energy and food during the period of the test and ignored the carbon content of the chemical, but this depends on the amount of inherent carbon they contained. Since the amount of 4-NP that was remaining at the end of the experiment was not determined exclusively, it may not be feasible to give a quantitative estimate of the amount that was used by the micro-organisms during the period of the test even though on visual examination, they seemed to have been exhausted.

For the solutions where a decrease in dissolved organic carbon at the end of the experiment was observed, the microorganisms might have used up the test chemicals and began to use their biomass as an exogenous carbon source. It has been reported that some species of microorganisms are capable of degrading phenolic compounds and utilizing same as the basic carbon and energy source in aerobic condition through a novel metabolic pathway [4], [21]. Research has also shown that wastewater organic carbon content could affect the structure and performance of the microbial community [5], [6], and that this may in turn alter micropollutant removal changes in the individual populations present [7].

4.4 Effect of CO_2 concentration on cell density

Figures 4.3 and 4.4 show initial and final CO_2 concentrations across all samples. From Table 4.4, there was significant increase ($p< 0.05$) in CO_2 concentration at the end of biodegradation across all test samples. This is an indication of microbial activity in these samples. Highest rise in the CO_2 was recorded for samples from Howden and Washington where end CO_2 66.81 and 69.34 mg C/l respectively. It was also observed that while Howden recorded substantial increase in cell density at the end of the biodegradation, sample from Washington showed 76 reduction in cell density. There was no change in the cell densities of sample from Cramlington despite increase in the CO_2 level. Sedgeletch showed similar increase as Cramlington in CO_2, but recorded substantial reduction (54%) in cell density.

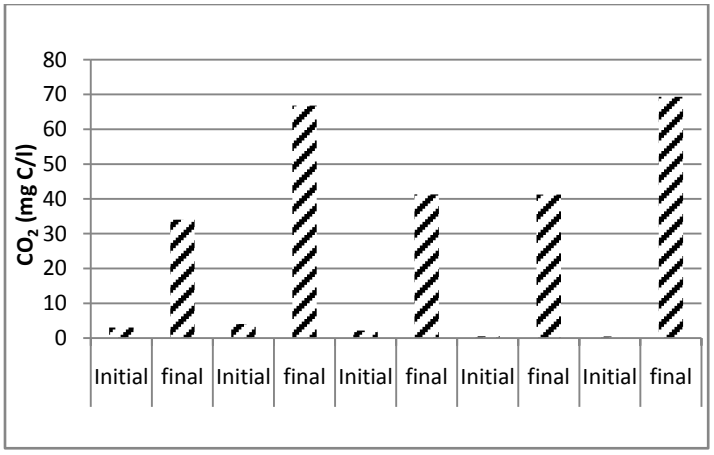


Fig. 4.3. CO_2 Concentration in test samples at beginning and end of biodegradation test

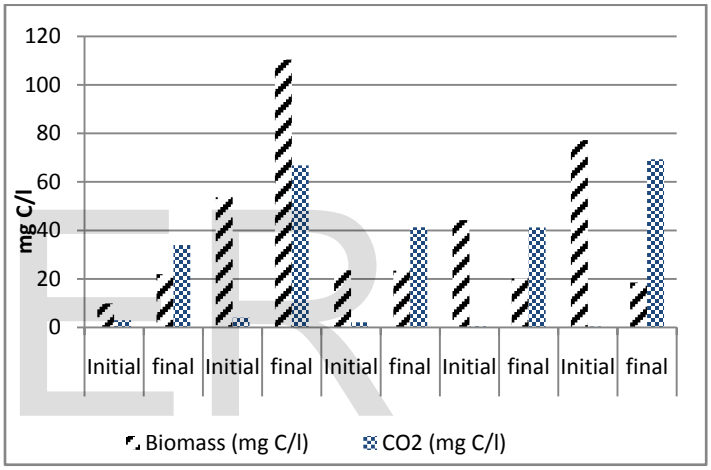


Fig. 4.4. Variation in biomass and CO_2 concentration of test samples before and after biodegradation

Table 4.4. ANOVA result of initial and final CO_2 concentrations from all WWTPs

Source	SS	df	MS	F	P
Treatment [between groups]	5883.0503	1	5883.0503	43.85	0.000166
Error	1073.3414	8	134.1677		
SS/Bl					
Total		9			

Decrease in cell density noticed in the media with increased CO_2 level could have resulted from acidification of the media following the dissolution of CO_2 in the aqueous media. Above

the saturation level of CO_2 in the headspace reagent bottle, excess CO_2 will dissolve in the aqueous media to form weak carbonic acid, which could be detrimental to the biological organisms, thereby leading to the death of many of them. On the contrary, there was biomass increase in the sample from Howden following substantial increase in the CO_2 level. The Howden plant has the highest effluent COD which has enabled the degrading organisms to be fairly acclimatized to "harsh condition". This condition could explain why the degrading microorganisms were able to multiply despite the huge increase in the CO_2 level.

5. Conclusion

This work has shown that 4-NP was biodegraded by activated sludge samples from different WWTPs in the Northeast of England. The findings imply that WWTPs could be employed in the treatment of chemicals that are considered hazardous to the environment. The chemicals could be utilized as a carbon source by micro-organisms, especially where the plant is predisposed to influent of relatively strong COD. However, the introduction of chemicals, such as 4-NP, can also result in reduced biological activity and death of micro-organisms in the treatment plant, especially where the treatment plant is not often exposed to influent of relatively strong concentration. It is, thus, necessary that periodic sampling be carried out in WWTPs if there is risk of chemical influent to ensure that biomass density is not hampered.

6. References

[1] American Public Health Association (1989). Standard Methods for the Examination of Water and Wastewater, in Lenore, S. C., Amold, E. G., & Rhodes, R. I. (Ed), 17thedn. *APHA*, Washington DC.

[2] Busca, G., Berardinelli, S., Resini, C., Arrighi, L. (2008). Technologies for the removal of phenol from fluid streams: a short review of recent developments. *Journal of Hazardous Materials*, 160, 265–288.

[3] Chaillana, F., Flecheb, A., Burya, E., Phantavonga Y-Hui, Saliot, A., Oudot, J. (2004). Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms.*Research in Microbiology,* 155 (7), 587 –595

[4] Corti, A., Frassinetti, S., Vallini, G., D'antone, S., Fichi, C. &Solaro, R. (1995). Biodegradation of nonionic surfactants. I. Biotransformation of 4-(1- nonyl) phenol by a Candida maltose isolate. *Environmental Pollution* 90 (1), 83–87.

[5] Docherty, K.M., Young, K.C., Maurice, P.A. &Bridgham, S.D. (2006). Dissolved organic matter concentration and quality influences upon structure and function of freshwater microbial communities.*Microb. Ecol.*, *52* (3), 378-388.

[6] Eiler, A., Langenheder, S., Bertilsson, S. &Tranvik, L. J. (2003).Heterotrophic bacterial growth efficiency and community structures at different natural organic carbon concentrations. *Appl. Environ. Microbiol.,69* (7), 3701-3709.

[7] Helbling, D.E., Johnson, D.R., Honti, M. &Fenner, K. (2012). Micropollutant biotransformation kinetics associate with WWTP process parameters and microbial community

characteristics. *Environ. Sci. Technol.*, *46* (19), 10579-10588.

[8] ISO Standard 14593, (1999). Water quality – evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium – method by analysis of inorganic carbon in sealed vessels (CO2 headspace test). *International Organization for Standardization*.

[9] Lee, K., & Merlin, F. X. (1999). Bioremediation of oil on shoreline environments: development of techniques and guidelines. *Pure and Applied Chemistry,* 71 (1), 161 – 171.

[10] Luo, Y., Zhang, H., Salerno, M., Logan, B. E., Bruns, M. A. (2008). Organic loading rates affect composition of soil-derived bacterial communities during continuous fermentative biohydrogen production. *Int. J. Hydrogen Energy*, *33* (22), 6566-6576.

[11] Martin, T.J., Goodhead, A., Snape, J. & Davenport, R. (2011). Impact of cell concentration methods on the performance of enhanced biodegradation tests within REACH.In SETAC Europe 21st Annual Meeting, 18th May 2011-Milan, Italy.

[12] Mcarthur. J. M., Algeo T. J., Van De Schootbrugge, B., Li Q., & Howarth, R. J. (2008). Basinal restriction, black shales, Re-Os dating, and the Early Toarcian(Jurassic) oceanic anoxic event. *Paleoceanography*, Vol. 23, PA4217 (1-22).

[13] OECD Guidelines for Testing of Chemicals (2006). 310 Ready biodegradability: CO2 in sealed vessels (headspace test). Paris: *Organization for Economic Cooperation and Development*. France.

[14] OECD (1992). OECD Guidelines for the Testing of Chemicals. Ready Biodegradability, Guidelines 301D. Guideline adopted 17 July 1992. *Organisation for Economic Cooperation and Development,* Paris, France.

[15] Okoh, A. I. (2006). Biodegradation alternative in the clean up of petroleum hydrocarbon pollutants.*Biotechnology & Molecular Biology*, 1, 38 – 50

[16] Ophardt, C. E. (2003). Acids and Bases.Virtual Chapbooks, Elmhurst College.http://www.elmhurst.edu/~chm/vchembook/184ph.html.

[17] Painter, H.A. (1995). Detail review paper on Biodegradability Testing. OECD series on the test guidelines programme no 2 Environmental monograph No. 98. Paris, France, Organization for Economic Cooperation and Development (OECD).

[18] Saelen G., Tyson R.V., Telnas N., & Talbot M. R. (2000). Contrasting watermass conditions during deposition of the Whitby Mudstone (Lower Jurassic) and Kimmeridge Clay (Upper Jurassic) formation UK.*Palaeoography*palaeoclimatology, palaeoecology, 163, 163-196

[19] Tan, D.T., Arnold, W.A. & Novak, P.J. (2013). Impact of Organic Carbon on the Biodegradation of Estrone in Mixed Culture Systems.*Environ. Sci. Technol.*, *47*(21), 12359-12365.

[20] Tomei, M. C., Rita, S., Angelucci, D. M., Annesini, M. C. &Daugulis, A. J. (2011). Treatment of substituted phenol mixtures in single phase and two-phase solid-liquid partitioning bioreactors.*Journal of Hazardous Materials*, 191(1-3), 190-195.

[21] Vallini, G., Frassinetti, S., D'andrea, F., Catelani, G. &Agnolucci, M. (2001). Biodegradation of 4-(1-nonyl)phenol by axenic cultures of the yeast Candida aquaetextoris: identification of microbial breakdown products and proposal of a possible metabolic pathway. *International Biodeterioration& Biodegradation* 47, 133–140.

[22] Volskay, V. T. Jr. & Grady, C. P. Jr. (1988). Toxicity of selected RCRA compounds to activated sludge microorganisms. *Journal of the water pollution Control Federation*, 55, 1850 – 1856.

[23] Bergheim, ., Helland, T., Kallenborn, R. &Kümmerer, K. (2010). Benzyl-penicillin (Penicillin G) transformation in aqueous solution at low temperature under controlled laboratory conditions. Chemosphere, 81 (11), 1477-1485.