



INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Peer Reviewed, Open Access, CrossRef Indexed Journal

ISSN (O) 2320-5407 | ISSN (P) 3107-4928A

 www.journalijar.com

 +91 92034 04180

MICROBIAL DEGRADATION OF SPARINGLY SOLUBLE DIETHYL PHTHALATE (DEP) AND DIBUTYL PHTHALATE (DBP) BY PURE MICROBES USING MICROCALORIMETRY

*A Thesis
Submitted to Graduate School in Partial
Fulfilment
of the Requirements for the Degree*

of
DOCTOR OF PHILOSOPHY
in
ENVIRONMENTAL SCIENCE
by

ALHAJI BRIMA GOGRA

Publisher: Jana Publication and Research LLP.

 Liberal Complex, 11/13 Pigdamber Rau, Indore, Madhya Pradesh-453331, India.

ISBN Number : 978-81-944253-4-2

Declaration of Statement of Originality



I, hereby, declare that the research reported in this dissertation is my original work and that all the sources or literature that I have used or quoted have been indicated and duly acknowledged by means of complete references. Therefore, this is the first report that has investigated the microbial degradation of sparingly soluble diethyl phthalate (DEP) and dibutyl phthalate (DBP) by pure microbes using microcalorimetry. The write-up is done by me under the supervision of Professor Dr. Jun Yao.

Signed: _____
(Candidate)

Date: _____

**CERTIFICATION BY MEMBERS OF THE
DISSERTATION COMMITTEE, SCHOOL OF
ENVIRONMENTAL STUDIES,
CHINAUNIVERSITY OF GEOSCIENCES**

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by **ALHAJI
BRIMA GOGRA** entitled “**Microbial Degradation of Sparingly Soluble Diethyl Phthalate (DEP) And
Dibutyl Phthalate (DBP) By Pure Microbes Using Microcalorimetry**” and recommend that it is accepted for
the award of the Degree of Philosophy.

Thesis approved by:

Chair

Dedication

With lots of love I heartily dedicate this work to:

My late parents (Rest in perfect peace. Amen/Ameen!!)

And

My wife (Madona), son (Moses) and daughter (Aminata) for your candid support and love.

ACKNOWLEDGEMENT

I would like to express my deepest gratitude and heartfelt thanks to my supervisor, Professor Dr. Jun Yao for her untiring patience and encouragement; and for giving me this opportunity to undertake this kind of research. Her guidance and mentoring, assistance and invaluable scientific advice were invaluable in the completion of the research.

This study would have been impossible without the contributions of other lecturers whom I consider as my co-supervisors (external supervisors): Professor Dr. Martin Ming Fat Choi of Hong Kong Baptist University, Hong Kong and Dr. Shixue Zheng and his research team members of Huazhong Agricultural University, Wuhan, who were very instrumental in every aspect of this work. Their dedications to this research, laboratory works and research data analyses were the foundations for the success of this project. I would also like to thank my colleagues (especially, Qian Yiguang for translating the abstract from English to Chinese) at the Key Laboratory of Biogeology and Environmental Geology of Chinese Ministry of Education, China University of Geosciences, Wuhan, for their assistance during the laboratory works.

This research program and studies would not have been fruitful without the generous scholarship support from the China Scholarship Council (CSC) of the People's Republic of China. A very big 'thank you' to them. My profound thanks to the staff of the International Cooperation Office (especially Mr. Hongtao Su, and Mr. Du) of China University of Geosciences, Wuhan. I would also like to thank the Government of Sierra Leone's Ministry of Education, Science and Technology for nominating me for the scholarship; and to the officials of Njala University, Freetown, Sierra Leone granting me study leave that enabled me pursue this study; and to my colleagues in the Department of Chemistry, School of Environmental Sciences who had to contend with heavier workload during the study period.

This work was also supported in parts by grants from National Outstanding Youth Research Foundation of China (40925010), International Joint Key Project from National Natural Science Foundation of China (40920134003), National Natural Science Foundation of China (40873060, 40673065), and International Joint Key Project from Chinese Ministry of Science and Technology, and the 111 Project (B08030).

Special and sincere thanks and appreciations extend also to my lovely wife (Madona), son (Moses) and daughter (Thomasia) for their unfailing love and support during the entire study period and also to my late parents (May the souls of both of you rest in permanent peace and may God's light perpetual on you, Ameen/Amen!) and relatives; Sierra Leoneans in China (especially in Wuhan), friends in Sierra Leone, PR China, for their inspirational support and untiring encouragement during my study period. Above all, my blessed thanks and appreciations go to the Almighty Allah for making everything possible for me in life. May He continue to bless me. Ameen/Amen!!!!!!!

Biosketch - Alhaji Brima Gogra

(I) Biodata:

Date of Birth: 03rd December, 1971

Place of Birth: Bo, Southern Province, Sierra Leone

Nationality: Sierra Leonean

Marital Status: Married (with two kids)

E-mail: abgogra@yahoo.co.uk / abgogra@gmail.com

(II) Educational Background:

September 2007 - July 2010: ***PhD degree candidate in Environmental Science (Engineering)(Distinction in Field Base Practice)***. ChinaUniversity of Geosciences, Wuhan.

Thesis: Microbial Degradation of Sparingly Soluble Diethyl Phthalate (DEP) and Dibutyl Phthalate (DBP) by Pure Microbes using Microcalorimetry. (Draft submitted on: 15th April, 2010)

November 2009 - March 2010: ***Cooperative research in Environmental Science***, Pennsylvania State University, University Park, Pennsylvania (PA), United States of America.

July 2002: ***Master of Science(M. Sc.) in Environmental Chemistry***. NjalaUniversityCollege, NUC (University of Sierra Leone, USL).

Thesis: A Comparative Study of the effects of some Environmental Unfriendly Practices on nearby watercourses in the Freetown Area.

July 2000: ***Bachelor of Science with Honors (B. Sc. Hons) in Environmental Chemistry(Distinction in Field Attachment)***. NjalaUniversityCollege, NUC (University of Sierra Leone, USL).

Thesis: The Effects of the Effluents from the Freetown Cold Storage Company Limited (FCSC) on the Georgebrook Stream and the surrounding soils.

(III) Employment History:

May 4, 2010 – Present ***Associate Editor***,

Continental Journal of Environmental Sciences (Online Journal). 2 Church Avenue, Oke-Eri Quarters, Oba-Ile.

P. O. Box 214, Akure, OndoState, Nigeria. Website link: <http://www.wiloludjournal.com/>

August 2006 – Present ***Lecturer II***,

Department of Chemistry, School of Environmental Sciences, Njala University, SLBS Building, New England, Freetown, Sierra Leone.

March 2003 – August 2006 ***Inspector of factories (Occupational Safety and Health)***,
Ministry of Labor, Social Security and Industrial Relations, New England, Freetown, Sierra Leone.

Sept. 2001 – March 2003 ***Teacher***,
United Methodist Secondary School for
Girls, Freetown, Sierra Leone.

Sept. 2000 – August 2001 ***Teacher***,
Harford School for Girls, Freetown, Sierra Leone.

May – September 1999 ***Field Attachment (Students' Internship)***,
Land and Water Development Division (LWDD) Laboratory, Ministry of Agriculture, Forestry and the
Environment, Tower Hill, Freetown, Sierra Leone.

January 1995 – Sept. 1995 ***Project Co-ordinator (Volunteer)***,
Futa Pejeh Community Development Project
Futa Pejeh, Pujehun District, Sierra Leone.

July – October 1993 ***Casual Clerk***,
Rokel Leaf Tobacco Development Company (RLTDC), Kamakwie, Bombali District, Sierra Leone.
September 1992 – August 1993 ***Assistant Teacher***, Browne Commercial Secondary School, Philip Street,
Wellington, Freetown.

(IV) Membership:

March 2009 – Present ***Member***,
International Students Organizing Committee
on Climate Change. China University of
Geosciences, Wuhan, PR China

October 1993 – Sept. 2002 ***Member***,
Environmental Sciences Students' Association (ESSA). Njala University College (University of Sierra Leone).

November 1993 – Present ***Member***,
Environmental Foundation of Sierra Leone (ENFOSAL), Freetown, Sierra Leone.

(V) Publications during PhD Study Programme:

A) Published papers/articles

- 1) **Alhaji Brima Gogra**, Jun Yao, Edward Hinga Sandy, Victor Tamba Simbay Kabba, Gyula Zaray. 2010. Trends in Solid Waste Management in Freetown, Sierra Leone. *Waste Management*, 30(5):938-939. **SCI Impact Factor (I.F.)= 2.208**
- 2) **Alhaji Brima Gogra**, Jun Yao, Victor Tamba Simbay Kabba, Edward Hinga Sandy, Gyula Zaray, Solomon Peter Gbanie, Tamba Samuel Bandagba. 2010. A Situational Analysis of Waste Management in Freetown, Sierra Leone. *Journal of American Science*, 6(5):124-135 (Core Journal publisher: Marsland Press, USA).
- 3) **Alhaji Brima Gogra**, Jun Yao, Edward Hinga Sandy, Gyula Zaray, Solomon Peter Gbanie, Celeste Tjobe, Tamba Samuel Bandagba. 2010. Evaluation of the Environmental Mitigation and Area Development (EMAD) component of the Bumbuna Hydroelectric Project in Sierra Leone. *Journal of American Science*, 6(6):51-64 (Core Journal publisher: Marsland Press, USA).
- 4) **Alhaji Brima Gogra**, Jun Yao, Edward Hinga Sandy, Shixue Zheng, Gyula Zaray, Bashiru Mohamed Koroma, Hui Zheng. 2010. Cell Surface Hydrophobicity (CSH) of *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger* and the Biodegradation of Diethyl Phthalate (DEP) via Microcalorimetry. *Journal of American Science*, 6(7):78-88 (Core Journal publisher: Marsland Press, USA).
- 5) **Alhaji Brima Gogra**, Jun Yao, Edward Hinga Sandy, Shixue Zheng, Gyula Zaray, Wei Fang Zhi. 2010. The study of the effects of microbial cell surface hydrophobicity on the degradation of dibutyl phthalate using microcalorimetry. *Journal of Environmental Science and Health, Part A. Toxic/Hazardous Substances and Environmental Engineering*. (Review process done; just **awaiting publication**. Publisher: Taylor & Francis). **SCI Impact Factor (I.F.) = 1.002**.

B) Papers/manuscripts under review process

- 1) **Alhaji Brima Gogra**, Jun Yao, Edward Hinga Sandy, Shixue Zheng, Gyula Zaray, Zejiao Luo. 2010. Cell surface hydrophobicity (CSH) of three microbial strains and n-octane biodegradation in the presence of diethyl phthalate (DEP). *Journal of Hazardous Materials*. **SCI Impact Factor (I.F.) = 2.975**.
- 2) **Alhaji Brima Gogra**, Jun Yao, Edward Hinga Sandy, Shixue Zheng, Gyula Zaray, Zejiao Luo. 2010. The degradation of n-octane in relation to cell surface hydrophobicity (CSH) and autoaggregation ability among microbial strains in the presence of dibutyl phthalate (DBP). *World Journal of Microbiology and Biotechnology*. **SCI Impact Factor (I.F.) = 0.945**
- 3) **Alhaji Brima Gogra**, Jun Yao, Edward Hinga Sandy, Shixue Zheng, Gyula Zaray, Zejiao Luo, Shiyun Cao. 2010. The microbial degradation of two phthalate acidic esters (PAEs) and their effects on the microbial cell surface hydrophobicity (CSH) of *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger* using microcalorimetry. *Toxicological and Environmental Chemistry*. (**Scholar ONE Manuscript Central**: Publisher: Taylor & Francis).
- 4) **Alhaji Brima Gogra**, Jun Yao, Edward Hinga Sandy, Martin Ming Fat Choi, Shixue Zheng, Gyula Zaray, Zejiao Luo, Shiyun Cao. 2010. The effects of Diethyl Phthalate (DEP) and Dibutyl Phthalate (DBP) on the Microbial Cell Surface Hydrophobicity (CSH) of *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus*

niger. *American Journal of Science*. **SCI Impact Factor (I.F.) = 2.27.**

5) Edward Hinga Sandy, Jun Yao, Shi Xue Zheng, **Alhaji Brima Gogra**, Huilun Chen, Hui Zheng, Thomas B. R. Yormah, Gyula Zaray, Brunello Ceccanti, Martin M. F. Choi. 2010. A comparative cytotoxicity study of isomeric alkylphthalates to metabolically variant bacteria. *Journal of Hazardous Materials*. **SCI Impact Factor (I.F.) = 2.975.**

C) Papers/manuscripts in preparation

1) A comparative study of the microbial degradation of sparingly soluble diethyl phthalate (DEP) and dibutyl phthalate (DBP) by pure microbes using microcalorimetry.

2) Toxicity evaluation of sparingly soluble diethyl phthalate (DEP) and dibutyl phthalate (DBP) on *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger* by microcalorimetric method.

(VI) Invited and Contributed Presentations in Conferences:

1) Jun Yao, Brunello Ceccanti, Ke Chen, Fei Wang, Huilun Chen, Rensheng Zhuang, Mohammad Russel, **Alhaji Brima Gogra**, Edward Hinga Sandy, Veronica Bianchi. 2009. Study of pollution and metabolic activities of microbial community in the sediment of Lake Honghu, Center-China by combined methods. A paper presented in the 7th International Symposium on Speciation of Elements in Biological, Environmental and Toxicological Sciences ISSEBETS/2009, Colloquium Spectroscopicum Internationale XXXVI – 2009, Budapest, Hungary – Pre-, Postsymposia, Special Sessions and Short Courses (27th August – 4th September 2009, Budapest, Hungary. Organizers: **GLADNET Training Committee**).

(VII) Recent Projects (October 2007 – April 2010):

I took part in the following projects as an investigator and/or a co-investigator:

- National Outstanding Youth Research Foundation of China (40925010).
- International Joint Key Project from National Natural Science Foundation of China (40920134003).
- National Natural Science Foundation of China (40873060, 40673065).
- International Joint Key Project from Chinese Ministry of Science and Technology.
- The 111 Project (B08030).

微量量热法研究纯微生物对难溶性的邻苯二甲酸二乙酯（DEP）和邻苯二甲酸二甲酯（DBP）的生物降解

摘要

大量的酞酸酯类化合物（PAEs）被人工合成而用来生产不同的塑料和广泛的分布于环境中。随着塑料大量的生产、使用和丢弃，在土壤圈（土壤、垃圾填埋地和淋滤液），水圈（自然水域、淡水、海水和地下水），大气圈和人类以及其他生物体的脂肪组织（这类化合物是亲脂性的）都发现了酞酸酯类这类化合物。这些难治性的水溶性有机化合物不仅在人类的一些慢性疾病的病原学上起着非常重要的作用，还能降低很多生物物种的生存能力，从而对自然生态系统中的大量物种都存在威胁。这些令人担心的发现已经唤起很多学者对环境中（特别是土壤和水中）的这类化合物的生物降解的浓厚兴趣。这类化合物的生物降解是影响环境中PAEs命运的关键过程。此研究的目的是利用微量量热技术等现代理化生综合方法去研究几种特定微生物，如细菌中的革兰氏阴性菌——大肠杆菌、革兰氏阳性菌——金黄色葡萄球菌以及真菌中的黑曲霉对两种可能有毒性的PAEs（DEP和DBP）的降解能力，探讨微生物与PAEs的相互作用机制，从而为修复和治理PAEs环境污染提供科学依据和技术支撑。

为了达到研究目的，本次研究做了如下一些特定的实验和得到相关结果：

1. 微量量热法

实验中利用TAM III微量量热仪测得了微生物生长速率常数 k ，抑制率 I 和半抑制率 IC_{50} 等参数的数值，而且相关系数 r 高达0.99。抑制率 I 是一个用来评价PAEs毒理效应非常重要的指标，而且是通过每一种微生物对相关的PAEs的参数计算出来的。

生长速率常数 k 的大小随着DEP和DBP的剂量的增多而减小，这也表明了这些化合物具有潜在的抗微生物活性即使在本次实验中它们被微生物全部消耗完了。实验中测得的在含有DEP介质中的半抑制率 IC_{50} （大肠杆菌， $197.37 \mu\text{g mL}^{-1}$ ；金黄色葡萄球菌， $154.43 \mu\text{g mL}^{-1}$ 和黑曲霉， $127.84 \mu\text{g mL}^{-1}$ ）表明黑曲霉和大肠杆菌分别是对DEP最敏感和最耐受的微生物；而在含有DBP的介质中的 IC_{50} （大肠杆菌， $154.43 \mu\text{g mL}^{-1}$ ；金黄色葡萄球菌， $137.95 \mu\text{g mL}^{-1}$ 和黑曲霉， $155.68 \mu\text{g mL}^{-1}$ ）表明金黄色葡萄球菌和黑曲霉分别是对DBP最敏感和最耐受的微生物。

研究发现生长速率常数随着PAEs的浓度增大而减小。当PAEs的浓度达到 $300 \mu\text{g mL}^{-1}$ 时，PAEs显示出了对微生物生长的完全抑制。在这种情况下，在量热实验过程中得到放热功率---时间曲线上看不到热效应。

不同微生物对DEP的降解效率 δ 分别为：大肠杆菌，51.9 - 93.5%；金黄色葡萄球菌，91.9 - 99.8%和黑曲霉，59.8 - 99.9 %。从而可以推断出金黄色葡萄球菌（平均降解效率 $\delta = 96.18 \%$ ）是降解DEP效率最高的微生物以及大肠杆菌（平均降解效率 $\delta = 83.83 \%$ ）是降解DEP效率最低的微生物。同样在对DBP降解实验中，降解效率 δ 分别为大肠杆菌，63.8 - 99.1%；金黄色葡萄球菌，13.7 - 98.5%和黑曲霉，92.3 - 100.0 %。因此表明黑曲霉（平均降解效率 $\delta = 97.27\%$ ）降解DBP效率最高的微生物而金黄色葡萄球菌（平均降解效率 $\delta = 75.95 \%$ ）降解DBP效率最低。

在DEP和DBP的含量较低和适中的实验中得到P-T曲线上第二个峰的高度比第一个峰的高度要高很多，但是在DEP和DBP的含量（分别为：200 和 300 $\mu\text{g mL}^{-1}$ ）比较高的实验中得到P-T曲线上两个峰值的高度差不多。因此，可以得出这样的结论：第一种方式的新陈代谢活动受到的影响比第二种方式的新陈代谢活动要大，而且在第二种新代谢活动中这些兼性厌氧微生物一定会消耗DEP或DBP并且紧接着会消耗那些在第一次降解DEP 和DBP过程中积累下来的降解中间产物（邻苯二甲酸单乙酯/邻苯二甲酸一丁酯、甲基亚乙基磷酸酯/磷酸一丁酯、邻苯二甲酸和原儿茶酸），这些中间产物是微生物继续生长一段时间需要的碳源和能量。PAEs（DEP 和 DBP）的生物降解遵循前人所提出的降解途径。

k 与初始浓度无关，而且*E. coli* + DEP, *S. aureus* + DEP, *A. niger* + DEP, *E. coli* + DBP, *S. aureus* + DBP, 和*A. niger* + DBP的平均一阶方程可以分别表示为： $\ln C = -0.00134t + A$, $\ln C = -0.00160t + A$, $\ln C = -0.00175t + A$, $\ln C = -0.00156t + A$, $\ln C = -0.00086t + A$, and $\ln C = -0.00200t + A$ ，相应的降解半衰期分别为： $t_{1/2}(\text{DEP}/E. coli) = 8.67 \text{ h}$, $t_{1/2}(\text{DEP}/S. aureus) = 7.54 \text{ h}$, $t_{1/2}(\text{DEP}/A. niger) = 10.92 \text{ h}$, $t_{1/2}(\text{DBP}/E. coli) = 9.66 \text{ h}$, $t_{1/2}(\text{DBP}/S. aureus) = 18.81 \text{ h}$, $t_{1/2}(\text{DBP}/A. niger) = 9.60 \text{ h}$ 。一阶的速率常数 k 逐渐减小表明DEP 和 DBP较高的初始浓度(200 and 300 $\mu\text{g mL}^{-1}$)阻碍了它们的生物降解。

这些微生物降解PAEs的能力看来与烷基侧链的长度有关。具有相对较短烷基侧链的DEP被降解的速率就比较长烷基侧链的DBP的速率高。很明显这些有机物的水溶性和微生物降解PAEs的能力随着辛醇和水的分配系数 k_{ow} 的增大而减小。微生物降解这两种PAEs能力的顺序为：DEP (*S. aureus*) > DBP (*A. niger*) > DEP (*A. niger*) > DEP (*E. coli*) > DBP (*E. coli*) > DBP (*S. aureus*)。通常，真菌黑曲霉具有非高的降解这两种PAEs的降解效率。

2. 分光光度计分析

微生物细胞表面的疏水性和自体凝集作用

没有摄入DEP 和 DPB的不同微生物的细胞表面的疏水性(*E. coli*, 64.90 %; *S. aureus*, 78.25 %; 和*A. niger*, 51.30 %)和自体凝集作用(*E. coli*, 63.00 %; *S. aureus*, 78.25 %; 和*A. niger*, 56.25 %)有很大的区别。在经过DEP 和DBP 处理后，*S. aureus*显示出了很强的细胞表面的疏水性和自体凝集作用，但*E. coli*和*A. niger*细胞表面的疏水性和自体凝集作用却比较温和。真菌(*A. niger*)的细胞表面的疏水性和自体凝集作用比细菌(*E. coli* and *S. aureus*)的弱，这说明真菌和细菌的表面组成和结构可能有着显著的差异。当培养基中加入一定量的DEP 和DBP(50 $\mu\text{g mL}^{-1}$)时，微生物细胞表面的疏水性[(*E. coli*, 43.85 %; *S. aureus*, 59.40 %; *A. niger*, 31.70 %) for DEP; (*E. coli*, 57.70 %; *S. aureus*, 65.95 %; *A. niger*, 43.10 %) for DBP] 和自体凝集作用 [(*E. coli*, 35.00 %; *S. aureus*, 58.00 %; *A. niger*, 23.00 %) for DEP; (*E. coli*, 43.25 %; *S. aureus*, 65.00 %; *A. niger*, 36.25 %) for DBP]

都有所增强。与空白进行比较时，这两种具有温和的细胞表面的疏水性的细胞比那些具有很强细胞表面的疏水性的细胞在自体凝集作用方面显示出了更大的减弱(DEP: *E. coli*, 44.44 %, *S. aureus*, 25.88 %, *A. niger*, 59.11 %; DBP: *E. coli*, 31.35 %, *S. aureus*, 16.93 %, *A. niger*, 35.56 %)。还发现所有微生物的细胞表面的疏水性都有差不多的减弱(DEP: *E. coli*, 32.90 %, *S. aureus*, 24.09 %, *A. niger*, 19.64 %; DBP: *E. coli*, 11.09 %, *S. aureus*, 15.72 %, *A. niger*, 15.98 %)。

微生物的细胞表面的疏水性和自体凝集作用的回归分析也得到了一个非常明显的线性正相关，而且DEP的相关系数为0.9584，DBP的相关系数为0.9968，这个线性关系和其他的文章所得到的线性关系非常吻合。

生物量和浊度

通过研究发现生物量和浊度之间存在线性关系。在加入PAE之后，浊度和生物量（log）随着培养时间的变化是呈指数形式的（遵循热动力学方程）而且与剂量无关。

即使*S. aureus*, *A. niger*和*E. coli*可以完全降解300 $\mu\text{g mL}^{-1}$ 的DEP和DBP，但是所需要的时间却比完全降解初始浓度比较低的这些有机物长得多。发现这三种微生物完全降解这两种PAEs所需要的时间是在同一个时间段里（大约84 -100 h）是非常有价值的。

实验结果也证明了生物量的浓度随着DEP和DBP初始浓度的增加而增大。此外，还发现了微生物在完全消耗掉DEP和DBP后还会继续生长一段时间，这也证明了在DEP和DBP的初始降解过程中积累下来的中间产物可以随后被用来支持微生物的生长。DBP的生物降解遵循一般的降解途径。

3. 色谱分析

气相色谱(Hewlett-Packard Model 5890; J.W., USA)所得到的结果表明DEP和DBP被微生物完全降解所需的最长时间分别为84 h和120 h。然而，生物降解DBP的速率比较慢。这种降解趋势与在微量量热实验和分光光度实验中得到的相似。这两种PAEs的生物降解速率看来与烷基侧链的长度有关，通过影响二酯的烷基官能团的长度来影响生物降解能力。

短链的DEP的降解速率比长链的DBP的降解速率高。这三种微生物都可以降解浓度高达300 $\mu\text{g mL}^{-1}$ 的DEP和DBP，但降解速率非常慢。实验中所用的两种酞酸盐的生物降解符合第一级反应模型。

这些微生物降解这两种PAEs能力的大小顺序为：DEP (*S. aureus*)> DBP (*A. niger*)> DEP (*A. niger*)> DEP (*E. coli*)> DBP (*E. coli*)> DBP (*S. aureus*)。这个顺序和用微量量热法所得到的结果一致，而且也符合其他学者所做的一些研究得到结论。总的说来，真菌（黑曲霉）是最有效的降解这两种PAEs的微生物。

实验中采用薄层色谱分析法测定了PAE的生物降解过程中的代谢产物。根据 R_f 值，发现降解过程所产生的中间产物分别是邻苯二甲酸单乙酯 (MEP)、邻苯二甲酸一丁酯 (MBP) 和邻苯二甲酸 (PA)。原儿茶酸盐，一种在微生物好氧降解DBP过程中非常重要的中间产物，在这次实验中没有检测到。这些结果表明MEP (来自DEP的降解) MBP (来自DBP的降解) 和PA是DEP和DBP水解的最终产物。

酞酸盐的生物降解途径的探讨有助于更好的弄清它们的矿化过程和他们代谢产物的毒理学行为。本次研究为将来的野外应用提供了一种合适的原位生物修复过程的模型（过程包括利用微生物降解环境污染物，生物修复的程度取决于降解微生物的多少、环境状况、污染物的种类以及生物降解微生物对这些污染物的可利用率），这种模型可以用来处理那些排放到环境的化合物。

总而言之，采用微量量热法来研究PAE的生物降解和PAE对微生物相应的刺激效应是非常可能和有效的。毫无疑问，微量量热法是一种研究微生物对PAEs的生物降解机理非常有用和精确的方法而且还能能为微生物学领域其他的学者提供非常重要的信息。

关键词：微量量热法 邻苯二甲酸二乙酯 邻苯二甲酸二丁酯 表面疏水性 降解效率 自体凝集作用

Microbial Degradation of Sparingly Soluble Diethyl Phthalate (DEP) and Dibutyl Phthalate (DBP) by Pure Microbes Using Microcalorimetry.

Abstract

Phthalic Acidic Esters (PAEs) are synthesized in massive amounts to produce various plastics and have become ubiquitous/widespread in environments and have been found in the pedosphere (soils, landfills and leachates), plants, hydrosphere (i.e. natural waters, fresh and ocean waters, groundwaters), the atmosphere, and human and other living organisms' fatty tissues (compounds are lipophilic/lyphophilic; i.e. fat-soluble) as a result of the production, usage, and disposal of plastics. Refractory water-soluble organic compounds perform a vital function in the etiology of certain chronic diseases in humans, reduce the viability of many plant and animal species, and pose a threat to one or more populations in natural ecosystems. Such worrying findings have aroused interest in the biodegradation of these compounds in soils and waters. Biodegradation of these compounds is a critical process affecting the environmental fate of PAEs. The sole aim of this study was to evaluate the microbial degradability of two potentially toxic PAEs (i.e., DEP and DBP) by two bacteria, (*Escherichia coli* and *Staphylococcus aureus*); and a fungus(*Aspergillus niger*) using microcalorimetryby monitoring their growth on media amended with/without DEP or DBP. To achieve this aim, the specific objectives were carried out and the results are presented:

1. Microcalorimetric method

The k , I and IC_{50} values were determined from the TAM III Microcalorimeter with correlation coefficients (R) greater than 0.99 (i.e., $R > 0.99$). Inhibitory ratio (I) is important index to evaluate toxicological effect of PAEs and was each calculated for each microbe in relation to these compounds. The k values, decreasing with increase in the DEP or DBP doses, show that these compounds had potential antimicrobial activity even though they were almost completely consumed in these experiments. The IC_{50} values (*E. coli*, 197.37 $\mu\text{g mL}^{-1}$; *S. aureus*, 154.43 $\mu\text{g mL}^{-1}$; and *A. niger*, 127.84 $\mu\text{g mL}^{-1}$) indicate that *A. niger* and *E. coli* are the most sensitive and most tolerant microbes to DEP, respectively; and those (*E. coli*, 154.43 $\mu\text{g mL}^{-1}$; *S. aureus*, 137.95 $\mu\text{g mL}^{-1}$; and *A. niger*, 155.68 $\mu\text{g mL}^{-1}$) indicate that *A. niger* and *S. aureus* are the most tolerant and most sensitive microbes to DBP, respectively.

It was observed that the growth rate constants decrease with the increasing concentrations of PAEs. When the concentrations of PAEs reached 300 $\mu\text{g mL}^{-1}$, the PAEs exerted complete inhibitory effects on the growth of the microbes, in this case no thermal effects can be observed in the power–time curve during the experimental time.

The DEP degradation efficiencies (δ) for *E. coli*, *S. aureus*, and *A. niger* are 51.9 - 93.5, 91.9 – 99.8 and 59.8 – 99.9 %, respectively; deducing that *S. aureus* (mean δ = 96.18 %) is the most efficient DEP degrader and *E. coli* (mean δ = 83.83 %) the least and those for DBP for *E. coli*, *S. aureus*, and *A. niger* are 63.8 – 99.1, 13.7 – 98.5 and 92.3 – 100.0 %, respectively; inferring that *A. niger* (mean δ = 97.27 %) is the most efficient DBP degrader and *S. aureus* (mean δ = 75.95 %) the least.

It is also interesting to note that the heights of the second peaks on the P-T curves increased greater than the first at low and medium DEP or DBP doses and little or diminishing peaks on curves for higher DEP or DBP doses (200 and 300 $\mu\text{g mL}^{-1}$). Therefore, it may be concluded that the first way of metabolism has been influenced greater than the second way and that in the second way of metabolism, these facultative microbes must have consumed the DEP or DBP and subsequently its related degradation intermediates (monoethyl phthalate/monobutyl phthalate, MEP/MBP; phthalic acid, PA; and protocatechuic acid) that may have accumulated during the primary degradation of DEP or DBP, as sources of carbon and energy needed for their continued growth for a definite time. Biodegradation of PAEs (DEP or DBP), respectively, follows the degradative pathway proposed somewhere.

The rate constants (k) were found to be independent of initial concentrations, and the mean first-order equations for *E. coli* + DEP, *S. aureus* + DEP, *A. niger* + DEP, *E. coli* + DBP, *S. aureus* + DBP, and *A. niger* + DBP could be expressed as: $\ln C = -0.00134t + A$, $\ln C = -0.00160t + A$, $\ln C = -0.00175t + A$, $\ln C = -0.00156t + A$, $\ln C = -0.00086t + A$, and $\ln C = -0.00200t + A$, respectively and their corresponding mean biodegradation half-lives were $t_{1/2}(\text{DEP}/E. coli) = 8.67 \text{ h}$, $t_{1/2}(\text{DEP}/S. aureus) = 7.54 \text{ h}$, $t_{1/2}(\text{DEP}/A. niger) = 10.92 \text{ h}$, $t_{1/2}(\text{DBP}/E. coli) = 9.66 \text{ h}$, $t_{1/2}(\text{DBP}/S. aureus) = 18.81 \text{ h}$, $t_{1/2}(\text{DBP}/A. niger) = 9.60 \text{ h}$, respectively. The first-order rate coefficient (k) decreased gradually, suggesting that the higher initial concentrations (200 and 300 $\mu\text{g mL}^{-1}$) of DEP and DBP hindered the biodegradation.

The biodegradability of the PAEs by these microbes appeared to be related to the length of the alkyl side chains. Relatively, the shorter-chain DEP was degraded at higher rate than the longer-chain DBP. It was observed that as the octanol/water partition coefficient (k_{ow}) increased and the water solubility decreased, the biodegradability of PAEs by these microbes declined. The order of the biodegradability of the two PAEs by these microbes was DEP (*S. aureus*) > DBP (*A. niger*) > DEP (*A. niger*) > DEP (*E. coli*) > DBP (*E. coli*) > DBP (*S. aureus*). Generally, *A. niger*, the fungus was found to be an efficient degrader of these two PAEs.

2. Spectrophotometric analyses.

Microbial Cell Surface Hydrophobicity and autoaggregation.

There were significant differences in CSH (*E. coli*, 64.90 %; *S. aureus*, 78.25 %; and *A. niger*, 51.30 %) and the autoaggregation abilities (*E. coli*, 63.00 %; *S. aureus*, 78.25 %; and *A. niger*, 56.25 %) amongst the microbes

without DEP or DBP treatment. *S. aureus* showed high while *E. coli* and *A. niger* showed moderate hydrophobicity and autoaggregation abilities in the absence of DEP or DBP treatment. The fungus (*A. niger*) displayed lower CSH and autoaggregation than that of the bacterial microbes (*E. coli* and *S. aureus*), illustrating that remarkable differences might exist in the bacterial and fungal surface composition and structure (*i.e.*, strain-specific characteristics). When DEP or DBP (50 $\mu\text{g mL}^{-1}$) was added to the culture media, there were decreases in both CSH [(*E. coli*, 43.85 %; *S. aureus*, 59.40 %; and *A. niger*, 31.70 %) for DEP; (*E. coli*, 57.70 %; *S. aureus*, 65.95 %; and *A. niger*, 43.10 %) for DBP] and autoaggregation ability [(*E. coli*, 35.00 %; *S. aureus*, 58.00 %; and *A. niger*, 23.00 %) for DEP; (*E. coli*, 43.25 %; *S. aureus*, 65.00 %; and *A. niger*, 36.25 %) for DBP] for the microorganisms. When compared with the blank (0.0 $\mu\text{g mL}^{-1}$), the two moderately hydrophobic cells recorded considerable reductions in autoaggregation ability (for DEP: *E. coli*, 44.44 %; *S. aureus*, 25.88 %; and *A. niger*, 59.11 %; and for DBP: *E. coli*, 31.35 %; *S. aureus*, 16.93 %; and *A. niger*, 35.56 %) than the strongly hydrophobic cell. Similar reduction in hydrophobicity for all microbes (for DEP: *E. coli*, 32.90 %; *S. aureus*, 24.09 %; and *A. niger*, 19.64 %; and for DBP: *E. coli*, 11.09 %; *S. aureus*, 15.72 %; and *A. niger*, 15.98 %) was also observed.

Regression analysis shows a significant positive linear correlation between hydrophobicity of microbial cultures and autoaggregation abilities of the microbes with $R = 0.9584$ for DEP and $R = 0.9968$, which is consistent with other literatures.

Biomass and Turbidity

The relationship between biomass and turbidity was observed to be linear. The changes in turbidity and biomass (log phase), as a result of PAE addition, with incubation time were found to be exponential [(hence, obeying the thermokinetic equation] and dose-dependent; and depict the phases of growth. Although 300 $\mu\text{g mL}^{-1}$ each of these compounds can also be degraded completely, the times needed for degradation were longer than the ones needed for lower initial concentrations of DEP or DBP for *S. aureus*, *A. niger* and *E. coli*, respectively. It's noteworthy that all three microbes completely degraded both PAEs within the same time period (*i.e.* about 84 to 100 h).

Research data also demonstrated that the biomass concentration increased with increasing initial DEP or DBP concentration. In addition, it was found that the microorganism continued to grow for a certain time after DEP or DBP was consumed, which indicated that intermediates accumulated during the primary degradation of DEP or DBP could subsequently be used for microbial growth. DEP and DBP biodegradations follow the general pathway.

3. Chromatographic analyses

Results from the GC (Hewlett-Packard Model 5890; J.W., USA) analyses showed that DEP and DBP were rapidly and completely degraded within 84 h (*i.e.*, 3.5 days) and 120 h (*i.e.*, 5 days) maximum, respectively. However, the degradation rates of DBP were slow. These degradation trends were also observed in the microcalorimetric

assays and the spectrophotometric methods. The biodegradation rates of these two PAEs appeared to be related to the length of the alkyl side chains, influencing the length of the alkyl group of the di-ester, and hence the biodegradability

The shorter-chain DEP was degraded at a higher rate than the longer-chain DBP. The three cultures were able to degrade actively up to 300 $\mu\text{g mL}^{-1}$ dose of DEP and DBP, however, at slower rates. The degradations of these two tested phthalates conform to the first-order reaction model.

The order of the biodegradability of the two PAEs by these microbes was observed to be in this order: DEP (*S. aureus*) > DBP (*A. niger*) > DEP (*A. niger*) > DEP (*E. coli*) > DBP (*E. coli*) > DBP (*S. aureus*). This trend was also observed in the microcalorimetric experiment, and also confirmed such observation made by other researchers. Generally, *A. niger*, the fungus was found to be the most efficient degrader of these two PAEs.

The metabolites of PAE biodegradation were identified by the Thin-layer chromatography (TLC). Based on the R_f values, the intermediates released into the medium were identified to be monoethyl phthalate (MEP), monobutyl phthalate (MBP) and phthalic acid (PA). Protocatechuate, a key intermediate during aerobic degradation of DBP via MBP and PA by numerous microorganisms, was not detected in this study. These results suggested that MEP (from DEP degradation) MBP (from DBP degradation) and PA were the ultimate products of DEP and DBP hydrolysis.

The degradation pathways exploration of phthalates possibly will help to understand their mineralization process and the toxicological behavior of their metabolites. This study is vital to tackle possible biodegradation concerns and to significantly contribute in the proper modelling of the *in situ* bioremediation processes (processes involving the use of microorganisms to degrade environmental pollution; the extent of bioremediation is critically dependent upon the presence of degrading microorganisms, the environmental conditions, the types of contaminants and the bioavailability of the contaminants to biodegradative microorganisms) for future field applications which could deal with the discharge of these compounds into the environment.

In conclusion, microcalorimetric investigations on the biodegradation and associated stimulatory effects of the PAE compounds on microorganisms are possible and promising. It is believed that microcalorimetry is a useful and accurate method for studying the complete biodegradation mechanism of microorganisms in the presence of the PAEs and can offer vital information for researches in the field of microbiology.

Keywords: microcalorimetry, diethyl phthalate, dibutyl phthalate, surface hydrophobicity, degradation efficiency, autoaggregation

List of Abbreviations and Acronyms

% *A_{Ag}* percent autoaggregation

% *Adh* percent adhesion

$\Delta S = S_0 - S_t$ change in concentrations of DEP or DBP between times 0 and *t*.

μW microwatts

A absorbance of the cultured media at 120 min intervals

A₁ absorbance measured at 660 nm of microbial culture after it's

washed twice with phosphate buffered saline (PBS) and resuspended in the same buffer.

A₂ absorbance of microbial suspensions due to microbial adhesion to n-octane at 660 nm using the spectrophotometer

ADHD *Attention Deficit Hyperactivity Disorder*

Al₂O₃ aluminum oxide (alumina)

A₀ absorbance of the cultured media at 0 min

BBP benzylbutyl phthalate

BBzP butylbenzyl phthalate

BOD Biological/biochemical Oxygen Demand

C concentration/dose (known)

CAS Chemical Abstracts Services

CDCP Centers for Disease Control and Prevention

CH₄ methane gas

CO₂ carbon dioxide gas

COD Chemical Oxygen Demand

CPPIU China Plastics Process Industry Union

CPSC Consumer Product Safety Commission

CPSIA Consumer Product Safety Improvement Act

CSH cell surface hydrophobicity

Danish EPA Danish Environmental Protection Agency

DBP dibutyl phthalate

DCP dicyclohexyl phthalate

DEHP di-2-ethylhexyl phthalate

DEP diethyl phthalate

DHP dihexyl phthalate

DIDP diisodecyl phthalate

DINP diisononyl phthalate

DMI dimethyl isophthalate

DMP dimethyl phthalate

DMT dimethyl terephthalate

DNA deoxyribonucleic acid

DnOP di-n-octyl phthalate

DOP dioctyl phthalate

DPP diphenyl phthalate

DPrP dipropyl phthalate

dQ/dt or P very small quantity of heat or the rate of heat production

dw dry weight

EASE Estimation and Assessment of Substance Exposure

EC European Commission

EDC endocrine disrupting chemical/compound

EMP ethyl-methyl phthalate

EU European Union

EUSES European Union System for the Evaluation of Substances

GC gas chromatography

H₂O water molecule

H₂O₂ hydrogen peroxide

He helium gas

I growth inhibitory ratio

IARC international agency for research on cancer

IC_{50} half inhibitory concentration of compounds (i.e. an inhibitory concentration capable of causing a 50% decrease of microbial growth rate)

k microbial growth rate constant

k_0 the growth rate constant of the blank (or control, 0.0 $\mu\text{g mL}^{-1}$)

k_c the growth rate constant of the PAE-containing samples at a concentration/dose C

K_{ow} octanol/water partition coefficient

LB medium Luria Broth medium

mA milliamps

MEHP mono-ethyl-hexyl-phthalate

N₂ nitrogen gas

NaCl sodium chloride

O₂ oxygen gas

$^{\circ}\text{C}$ degree centigrade

OD₆₆₀ optical density measured spectrophotometrically at 600 nm

PA phthalic acid

PAE phthalic acidic ester

PBS phosphate buffered saline

PDEs phthalate di-esters

PETE polyethylene terephthalate ethylene

P_{Max} peak-heat output power

PMEs phthalate monoesters

PPARs peroxisome proliferator-activated receptors

PSM Potato sucrose medium

P-T curve power-time or thermogenic curve

PVC polyvinyl chloride

Q_{rt} total heat generated or total thermal effect

REACH Registration, Evaluation, Authorization and Restriction of Chemical substances

R correlation coefficient

R_f the *ratio* of substance advance distance to solvent advance distance (i.e. substance advance distance/solvent advance distance)

S_0 concentrations (moles) of DEP or DBP at time 0.

SCHER Scientific Committee on Health and Environmental Risks

SiO₂ silicon dioxide (silica gel)

S_t concentrations (moles) of DEP or DBP at time t .

TA terephthalic acid

TAM III the third generation multi-channeled thermal activity monitor

t_G generation time of microbial growth

TLC thin-layer chromatography

t_{Max} peak-time, related to the maximum position on the P-T curves

UASB upflow anaerobic sludge blanket

USEPA US Environmental Protection Agency

USNRC US National Research Council

W watts

WHO World Health Organization

WWTP waste water treatment plant

δ relative rate of degradation

Table of contents

CHAPTER ONE: INTRODUCTION	22
§1.1 GENERAL BACKGROUND.....	23

§1.2 JUSTIFICATION OF STUDY.....	25
§1.3 AIM AND OBJECTIVES OF STUDY.....	28
§1.4 SIGNIFICANCE/RATIONALE OF THIS STUDY.....	28
§1.5 THESIS CONTENTS AND STRUCTURE.....	29
CHAPTER TWO: LITERATURE REVIEWS.....	29
§2.1 GENERAL BACKGROUND TO PHTHALIC ACIDIC ESTERS (PAEs).....	30
2.1.1 <i>History</i>	30
2.1.2 <i>The basic chemical structure of Phthalates</i>	30
2.1.3 <i>Uses of phthalates</i>	30
2.1.4 <i>Production</i>	31
2.1.5 <i>Properties</i>	32
2.1.6 <i>Distribution and Bioaccumulation</i>	32
2.1.7 <i>Health effects</i>	32
2.1.8 <i>Legal status</i>	37
2.1.9 <i>Identification in plastics</i>	38
§2.2 ECOTOXICOLOGY AND ENVIRONMENTAL CHEMISTRY OF PAEs.....	38
2.2.1 <i>Assessment of PAEs Exposure</i>	38
2.2.2 <i>The biodegradability of phthalates</i>	40
§2.3 RELATED REVIEWS ON INSTRUMENTATIONS.....	47
2.3.1 <i>Microcalorimetry</i>	47
2.3.2 <i>Chromatographic Techniques</i>	48
§2.4 REVIEW OF LITERATURES RELATED TO TEST MICROORGANISMS.....	52
2.4.1 <i>Escherichia coli (E. coli)</i>	53
2.4.2 <i>Staphylococcus aureus (S. aureus)</i>	53
2.4.3 <i>Aspergillus niger (A. niger)</i>	54
CHAPTER THREE: METHODOLOGY.....	55
§3.1. MATERIALS AND METHODS.....	55
3.1.1 <i>Materials</i>	55
3.1.2 <i>Methods</i>	56
§3.2 STATISTICAL ANALYSES.....	61
CHAPTER FOUR: RESULTS AND DISCUSSIONS.....	62
§ 4.1 MICROCALORIMETRY.....	63
4.1.1 <i>The growth thermogenic curves at various doses of DBP</i>	63
4.1.2 <i>Inhibition and Tolerance</i>	64
4.1.3 <i>Degradation Efficiencies</i>	65
4.1.4 <i>DEP and DBP Biodegradation Kinetics</i>	70
§ 4.2 SPECTROPHOTOMETRIC EXPERIMENTS.....	77
4.2.1 <i>Spectrophotometric assays</i>	77
§4.3 CHROMATOGRAPHIC ANALYSES.....	87
4.3.1 <i>DEP and DBP degradation Time courses and cell growth by different microbes</i>	87
4.3.2 <i>Identification of metabolites of PAE biodegradations</i>	88
§4.4 CHAPTER SUMMARY.....	90
CHAPTER FIVE: SUMMARY, GENERAL CONCLUSIONS AND RECOMMENDATIONS.....	92
§5.1 SUMMARY.....	93
§5.2 GENERAL CONCLUSIONS.....	95
§5.3 RECOMMENDATIONS.....	97
REFERENCES.....	118
APPENDICES.....	145

LIST OF TABLES

Table 2.1. Table showing the most commonly found phthalates	17
Table 2.2. Selected properties of DEP and DBP (Adapted from Staples <i>et al.</i> , 1997).	20
Table 2.3: Types of Chromatographic techniques used in this research	48
Table 4.1. Enumerative microcalorimetric experimental results of the effects of increasing doses of DEP on the growth of the three microbial strains at 28 °C	75
Table 4.2. Enumerative microcalorimetric experimental results of the effectsof increasing doses of DBP on the growth of the three microbial strains at 28 °C	78
Table 4.3. Half-lives and kinetic equations of the DEP and DBP biodegradationsby <i>E. coli</i> , <i>S. aureus</i> and <i>A. nige</i>	79
Table 4.4. Metabolites (degradation products) of DEP and DBP degradations under denitrifying conditions	95
Table 4.5. Means of the calculated % Inhibition and IC_{50} ($\mu\text{g mL}^{-1}$) values from OD_{660} values	96

LIST OF FIGURES

Fig. 4.1. The growth thermogenic (power-time, P-T) curves of (a) <i>E. coli</i> in LuriaBroth (LB) medium, (b) <i>S. aureus</i> in LB medium and (c) <i>A. niger</i> in potato sucrose medium (PSM) affected by varied doses of DEP at 28 °C	70
Fig. 4.2. The growth thermogenic (power-time, P-T) curves of (a) <i>E. coli</i> in Luria Broth (LB) medium, (b) <i>S. aureus</i> in LB medium and (c) <i>A. niger</i> in potato sucrose medium (PSM) affected by varied doses of DBP at 28 °C	72
Fig. 4.3. Relationship between the microbial growth rate constants (<i>k</i>) of the three microbesand the various concentrations of DEP [(a), (b), (c)]; and DBP [(d), (e), (f)]	74
Fig. 4.4. Surface Hydrophobicity of the three microbial strains grown in their respective medium only or a medium containing DEP and DBP.....	83
Fig. 4.5. Autoaggregation ability of the three microbial strains grown in their respective medium only or a medium containing DEP and DBP.....	84
Fig. 4.6. Autoaggregation kinetics of the three microbial strains (to n-octane) grownin their respective medium only or a medium containing (a) DEP and (b) DBP	85
Fig. 4.7. The correlation between autoaggregation ability and the hydrophobicity of the microbes in medium containing (a) DEP and (b) DBP.....	86
Fig. 4.8. The correlation between the microbial cell surface hydrophobicity and (a) mean DEP degradation efficiency and, (b) inhibition (<i>IC</i> ₅₀).....	87
Fig. 4.9. The correlation between the microbial cell surface hydrophobicity and (a) mean DBP degradation efficiency and, (b) inhibition (<i>IC</i> ₅₀).....	88
Fig. 4.10. Biomass dry weight/Growth curves of the three microbes influenced by 200 µg mL ⁻¹ a) DBP and b) DEP at 28 °C	89
Fig. 4.11. Turbidity changes at 660nm (OD ₆₆₀) with incubation time of the three microbes in the absence (blank) and presence of the various concentrations of DEP [(a), (b), (c)]; and DBP [(d), (e), (f)].....	90
Fig. 4.12. Degradations by the three strains at various initial concentrations of DEP [(a), (b), (c)] and DBP [(d), (e), and (f)] by the GC measurements.....	94

CHAPTER ONE: INTRODUCTION

This part discusses the background of the study, presents the topic of the dissertation, points out its importance and significance, highlights the problem area(s) to be addressed and states the goals of research or sets out key objectives to be followed.

§1.1 General Background

Phthalate esters or Phthalic acidic esters (PAEs) are a group of multifunctional industrially important sparingly soluble organic compounds or chemicals (known technically as dialkyl or alkyl aryl esters of 1,2-benzenedicarboxylic acid) widely applied, since the last half of last century, in a variety of consumer (non-reactive plasticizers, shoes, insect repellents, synthetic fibres, plastics, weather stripping, paints, blood bags and their tubings, traffic cones, toys, vinyl gloves, flea collars, conveyor belts used in food packing operations, etc) and personal care products (cosmetics) as additives that improve the mechanical properties (i.e., workability) and flexibility of these polymeric materials. Phthalates are manufactured in large amounts (with a global scale increase from 2 to 2.5 million tons in the production of phthalates been recorded from 1980 to 2000) most importantly to produce various forms of plastics (Zeng *et al.*, 2008; Liang *et al.*, 2008). Recent consumption rate of PAEs in China is above 0.87 million tones year⁻¹ (China Plastics Process Industry Union, CPPIU; China Plastics Industry Yearbook, 2004; cited in Zeng *et al.*, 2008) and the global production is approximately 6.0 million tones (Mackintosh *et al.*, 2006). PAEs are chiefly used as plasticizers in polyvinyl chloride production (Zeng *et al.*, 2008).

Six (6) of the twenty (20) PAEs produced for commercial purposes [dimethylphthalate (DMP), diethylphthalate (DEP), di-n-butylphthalate (DBP), di-2-ethylhexylphthalate (DEHP), dioctylphthalate (DOP) and dipropylphthalate (DPP)] are considered as priority pollutants by the United States Environmental Protection Agency (Keith and Telliard, 1979; US-EPA, 1982) and People's Republic of China (European Union Risk Assessment Report, 2003; Liang, 2008; Zeng *et al.*, 2008). These compounds are included in the "priority list of substances for further evaluation of their role in endocrine disruption" by the European Commission (Groshart and Okkerman, 2000). Among these six PAEs, DEP and DBP are amongst the most frequently identified PAEs in environmental matrices (Kleerebezem *et al.*, 1999).

DEP and DBP are ubiquitous aromatic hydrocarbon contaminants and are considered to be hazardous as a result of their mutagenic, hepatotoxic and carcinogenic properties (Colón *et al.*, 2000; Hauser *et al.*, 2004; Main *et al.*, 2006). Many researchers have reported that environmental degradation of PAEs could occur by hydrolysis, photodegradation and biodegradation (Staples *et al.*, 1997; Zeng *et al.*, 2003; Jianlong *et al.*, 2004; Zhou *et al.*, 2005). Metabolic breakdown of PAEs by microorganisms has been carried out both in aerobic (O'Grady *et al.*, 1985; Zeng *et al.*, 2003) and anaerobic processes (Shelton *et al.*, 1984; Chauret *et al.*, 1996); and degradation studies have been carried out in soil (Jianlong *et al.*, 2004; Zhou *et al.*, 2005); fresh water (Johnson and Heitkamp,

1984); activated sludge (Sugatt *et al.*, 1984; Wang *et al.*, 1997); and sediments (Johnson and Heitkamp, 1984; Painter and Jones, 1990). DBP was also removed from solutions by adsorption onto various adsorbents including activated carbon materials (Herbert and Zeng, 2004; Bodzek *et al.*, 2004; Adhoum and Monser, 2004; Ayranci and Bayaram, 2005). The general biodegradation pathway for PAEs in environment was reported (Staples *et al.*, 1997; Liang *et al.*, 2008).

Miyoshi (1895) was the first to describe the ability of microorganisms to utilize organic compounds as sole carbon source, *i.e.*, microbial utilization of paraffins. A great diversity of different microbes (bacteria, fungi, yeast, and moulds) is capable of degrading organic compounds (like PAEs, and other oily-liquids) in the environment. Bacteria belong to a group of the 'best bio-degraders' of organic compounds (Olivera *et al.*, 2003; Chao *et al.*, 2006) including oily-liquids and other hydrocarbons. Unfortunately, little or no information is available on the microbial degradation of these compounds with focus on the relationship between the extent of degradation achieved and cell surface hydrophobicity (CSH), which seems to be an avoided topic especially in systems containing more than one carbon source.

Many researchers have tried to proffer the definition of hydrophobicity: the thermodynamists define it from a thermodynamic view (Edsall, 1992) and the microbiologists define it from a phenomenological view (Kauzmann, 1959; Doyle, 2000). Breslow (1991) observed that, hydrophobicity is the tendency of apolar species to aggregate in aqueous medium so as to decrease the hydrocarbon-water/oil-water interfacial area. The inability of non-polar compounds like phthalic acid esters (PAEs) to dissolve in aqueous medium has been associated with the hydrophobicity of these compounds, in fact, making the term misleading; the London dispersion interactions between molecules of water and non-polar compounds are favorable and quite substantial. Reviews by Karplus (1997) and Doyle (2000) attempted to throw light on hydrophobicity in terms of both biology and chemistry. Adherence of a microorganism to a surface may occur through hydrophobic effect provided the associating sites have sufficiently high densities of non-polar areas. Microorganisms, including bacteria and fungi have adopted ways to use the hydrophobic effect to adhere to surfaces. Hydrophobic microorganisms, in particular, are capable of adhering to the oil-water interface, and of utilizing oil components as a source of energy for growth and metabolism (Marshall, 1991). Duncan-Hewitt (1990) observed that there are justifying reasons to believe that the hydrophobic effect (attributed to cell wall proteins and lipids) may be the driving force for the adhesion of most microorganisms.

Microbial cell surface hydrophobicity (CSH) is of paramount importance for cell survival because it controls the process of association between the cells and other surfaces, thereby, playing an important role in degradation of hydrophobic substrates. As the cell surface becomes more hydrophobic, the association of the cell with the hydrophobic substrate becomes stronger, thereby resulting in an increase in the degradation rate (Al-Tahhan *et al.*, 2000; Prabhu and Phale, 2003). The rate of biodegradation of organic compounds is dependent upon several physicochemical and biological parameters. For instance, PAEs with shorter alkyl-chains like diethyl phthalate

(DEP) and dibutyl phthalate (DBP) are relatively easily biodegraded than phthalate esters with longer alkyl-chains such as di-(2-ethylhexyl)-phthalate (DEHP) (Chang *et al.*, 2004; Chao *et al.*, 2006). So it is expected that DEP is more easily biodegraded than DBP. The poor solubility of PAEs in water is potentially one of the commonest physicochemical properties which hinder their rates of biodegradation in the environment and this makes PAEs poorly bio-available; thus hampering their degradations (Rosenberg *et al.*, 1992; Bouchez *et al.*, 1995; Efroymson and Alexander, 1995; Churchill *et al.*, 1999). Bioavailability of hydrophobic organic compounds (a physicochemical parameter critical in the overall rate of compound degradation) is a function of phase solubility and solution transport processes. For chemical and physical compatibility between and/or among the organisms and the hydrophobic DEP, or DBP and *n*-octane substrates, the microbes must possess cell surface hydrophobicity; hence, leading to enhanced mutual interactions between and/or among them (Busscher *et al.*, 1995), increased dispersion and greater bioavailability of DEP and/or DBP substrates, and enhanced utilization.

§1.2 Justification of Study

Refractory water-soluble organic compounds perform a vital function in the etiology of certain chronic diseases in humans, reduce the viability of many plant and animal species, and pose a threat to one or more populations in natural ecosystems. PAEs are synthesized in massive amounts to produce various plastics and have become ubiquitous/widespread in environments and have been found in the pedosphere (soils, landfills and leachates) (Schwarzbauer *et al.* 2002; Zheng *et al.* 2007), plants, hydrosphere (i.e. natural waters, fresh and ocean waters, groundwaters), the atmosphere (Wensing *et al.* 2005), and human and other living organisms' fatty tissues (compounds are lipophilic/lyphophilic; i.e. fat-soluble) (Pye and Patrick, 1983; Gibbons and Alexander, 1988) as a result of the production, usage, and disposal of plastics (Staples *et al.* 1997). Such worrying findings have aroused interest in the biodegradation of these compounds in soils and waters. Unlike the aliphatic hydrocarbons, however, surprisingly little information is available on the biodegradation of water-insoluble organics. This rarity of such data is of concern to regulatory agencies, which have encouraged researches on the biodegradation of environmental pollutants that are present in nature at levels that exceed their aqueous solubility (Boethling, 1984).

These compounds, when used as plasticizers, are not chemically bonded to the plastics polymer and as a result, can ultimately migrate from the plastics into the environment. Several of these phthalates detected in these environments (Pye and Patrick, 1983; Boethling, 1984) have become environmental concerns because of their hepatotoxicity, teratogenicity, and carcinogenicity in nature (Matsumoto *et al.* 2008), and are also endocrine-disrupting or –modulating; causing some of the PAEs to be placed on the list of priority pollutants of the US Environmental Protection Agency (USEPA). Humans are exposed to these compounds through ingestion, inhalation, and dermal exposure on a daily basis. Epidemiological studies in test animals indicate an increase in some kind of cancer, behavioral changes, anomalies in the reproductive and immunologic functions of some species (Rivas *et al.*, 1997). Their uptake by microorganisms may involve an initial cleavage to yield water-soluble products by extracellular enzymes, direct surface contact, or microbially enhanced solubilization (Reddy *et al.*, 1983; Zilber *et al.*, 1980), or the organisms may utilize only the soluble form of the substrate so that

utilization of the insoluble form coincides with spontaneous dissolution. Moreover, any combination of these mechanisms is possible.

The effects that these compounds may pose to the environment have lead to many studies to evaluate the real impact, especially towards fish, and mollusks (Hylland and Haux, 1997). But, in 1996, the World Health Organization (WHO) and the European Union (EU) initiated a programme for the evaluation of human health risks due to the widespread presence of endocrine disrupting compounds (EDCs) in the environment (Groshart and Okkerman, 2000). These compounds have been detected in sewage sludge, sediments, surface and groundwater (Field and Reed, 1996; Castillo and Barceló, 1999; Petrovic and Barceló, 2000). Since the world awareness of the human uptake of these compounds through diet (Guenther *et al.*, 2002), efforts is given to survey food matrices, including drinking water either from distribution or bottled, to diminish human exposure. A major issue is to generate data which permits to better evaluate the levels of EDCs in these matrices for a better risk assessment. The presence of EDCs in foods is attributed mainly to migration from the packing material (Summerfield *et al.*, 1998; Brotons *et al.*, 1995). Just as in any other types of food, water may contain phthalates and other types of EDCs as a result of migration of such compounds from the epoxy resins or paints used as inner coating materials in water reservoirs and pipes (Romero *et al.*, 2002).

Among other phthalates, two low molecular weight phthalate esters (DEP and DBP), which are commonly used as additives in the plastic industries and exhibit the highest aquatic toxicity (Moore, 2000), were chosen in the present work because they can be present in material which is in contact with food and beverages (Hojslev and Breindahal, 2000; Balafas *et al.*, 1999). These compounds are included in the “priority list of substances for further evaluation of their role in endocrine disruption” by the European Commission (Groshart and Okkerman, 2000).

Numerous studies indicated that phthalates can be degraded by bacteria and fungi under aerobic, anoxic, and anaerobic conditions. Microbial degradation of PAEs has been regarded as one of the principal ways of environmental degradation for these toxicants because of their low rate of chemical hydrolysis and photolysis (i.e., long time periods), and low percentage degradation and quantities of PAEs used by other methods (Staples *et al.*, 1997; Zeng *et al.*, 2003) and the corresponding formation of toxic metabolites and/or products. These one benzene ring containing compounds can be degraded by countless number of microorganisms existing in the natural environment (Kim *et al.*, 2002; Yuan *et al.*, 2002; Hashizume *et al.*, 2002; Catwright *et al.*, 2000). The complete mineralization of the aromatic hydrocarbon yields products such as cell mass and inorganic materials, including CO₂ and H₂O, considerably harmless.

The ubiquitous occurrence of hydrophobic cell surface property among PAE-degrading population of microorganisms will be a favorable feature for DEP and DBP contamination bioremediation activity in any environmental matrix. Potentially, the rate-limiting step in biodegradation of hydrophobic compounds is the ability of the compounds to be solubilized and transported into microbial cells capable of metabolizing them.

Though there exist among microorganisms, direct contact and DEP- or DBP-solubilization mechanisms for substrate uptake but their relative contribution in a population of PAE degraders is yet unknown.

Unfortunately, not many researchers have dealt with the quantitative metabolism of PAEs. Recently, a few investigators have begun to make quantitative balances, demonstrating the energy relationships in the metabolism of PAE compounds. More research is definitely needed into the energy relationships for the metabolism of the different PAE compounds. However, there is no existing published thermodynamic and kinetic data on the biodegradation of DEP and DBP using microcalorimetry. This study was undertaken to determine the thermodynamic and kinetic parameters of the biodegradation of DEP and DBP *Escherichia coli*, and *Staphylococcus aureus*) and *Aspergillus niger*.

With the advent of microcalorimetric techniques in the past recent decades, the studies of living materials have been greatly improved. A microcalorimeter has been successfully employed to elucidate the effects of various substances and culture conditions on microbial metabolism (Zheng *et al.*, 2006; Chen *et al.*, 2007; Ma *et al.*, 2007; Yao *et al.*, 2007; Yao *et al.*, 2008; Wang *et al.*, 2008; Chen *et al.*, 2008). Microcalorimetry is a highly sensitive method to assess the overall biomass and activity of living microorganisms. Temporary details (such as thermal effect, etc) not observable by other techniques and other much useful information (or important indices), both qualitative and quantitative (like microbial growth rate constant, heat yield of microbial growth, etc), can also be revealed by microcalorimetric method. Metabolic activities of living organisms result in a final exothermic effect in nature (Prado and Airolidi, 1999). In this respect, microcalorimetry is one of the techniques that can be applied to study metabolic activities of microorganisms (Zheng *et al.*, 2006; Ma *et al.*, 2007). This analytical method has been confirmed to be valid as an alternative in the investigation of substrate metabolism and microbial growth, as it allows the continuous monitoring of the activity of a living process *in situ* for a prolonged period without disturbing the system (Wadsö, 1980; Barros *et al.*, 1999; Núñez-Regueira *et al.*, 2006). Wide ranging practical applications of such a technique have been envisioned (Wadsö, 1980).

Taking into consideration the significance of phthalates and the more general question of the mechanisms of degradation of sparingly soluble compounds by microorganisms, this study was undertaken, using mainly, microcalorimetry and other analytical methods, to determine: the means by which microorganisms carry out the degradation of these sparingly soluble esters, their respective PAEs biodegradation efficiencies, and the biodegradation metabolites/products formed.

To the best of my memory, after going through all previous works done (published or unpublished) on PAE degradation or toxicity, it was realized that nobody has ever used microcalorimetry to accomplish such research tasks. This work is anticipated to provide significant directions for the study of PAE biodegradation; and hence, possible PAE clean-up in the environment.

§1.3 Aim and Objectives of Study

The sole aim of this study was to evaluate the microbial degradability of two potentially toxic PAEs (i.e., DEP and DBP) by three microbes (two bacteria, *Escherichia coli* and *Staphylococcus aureus*; and a fungus, *Aspergillus niger*) using microcalorimetry by monitoring their growth on media amended with/without DEP or DBP. To achieve this aim, the following specific objectives were carried out:

- (a) Investigating the target organisms for their cell surface properties, including cell autoaggregation ability, hydrophobicity, etc, as these properties determine their survival by controlling the process of association between the cells and other surfaces, thereby, playing an important role in degradation of hydrophobic substrates;
- (b) Using the TAM III microcalorimeter (as the main research tool/instrument) to determine the respective degradation abilities of these two organic compounds by these test microbes; and to develop kinetic equations of the reactions taking place in the microcalorimeter at a constant temperature of 28 °C and heater pulse power of 120 μ W.
- (c) Also serving as confirmatory tests, a gas chromatography GC will be used to evaluate the degradation efficiencies of each of these PAEs by these microorganisms; and to identify the residues (or metabolites) of these PAE degradations using thin-layer chromatography (TLC);
- (d) To validate the results got from the above experiments, traditional physico-microbial tests, like shake flask experiments, will be carried out to determine the biomass and optical density (OD)/turbidity using spectrophotometry.

§1.4 Significance/Rationale of this Study

Assessment of the PAE biodegradation potentials of these three wild type cells of microorganisms is a vital tool for the characterization of environmental samples. This research is employing this set of organisms for the first time in PAEs degradation using a recent modern analytical technique to evaluate their degradation efficiencies will significantly contribute to the field of ecotoxicology with respect to the metabolism of these PAEs in environmental monitoring and will also help tackle possible contamination/pollution concerns and to suitably replicate the *in situ* bioremediation practices for future field applications which could control the discharge of these compounds into the environment and their concentrations therein.

This research will help to evaluate the microbial cell surface property of the test organisms; to investigate if there is any effect of PAE treatment on the determined property; and to establish if there exists any relationship between CSH and PAE biodegradation. The present work also investigates the phthalates degradation potentialities of three microbes and includes the following aspects: the relationship between the chemical structures of phthalates and their biodegradability; the biodegradation of phthalates by pure cultures; the biodegradation of phthalates under aerobic and anaerobic experimental conditions; and the biodegradation pathways of phthalates by identifying the degradation metabolites. The degradation pathways exploration of phthalates possibly will help to understand

their mineralization process and the toxicological behavior of their metabolites. This study is vital to tackle possible biodegradation concerns and to significantly contribute in the proper modelling of the *in situ* bioremediation processes (processes involving the use of microorganisms to degrade environmental pollution; the extent of bioremediation is critically dependent upon the presence of degrading microorganisms, the environmental conditions, the types of contaminants and the bioavailability of the contaminants to biodegradative microorganisms) for future field applications which could deal with the discharge of these compounds into the environment.

Principally, this study is like a pioneer work in the fields of environmental remediation and ecotoxicology, utilizing these species and microcalorimetry as research tools. The data from this research will be of immense significance to regulatory agencies (which have been encouraging researches on the biodegradation of environmental pollutants that are present in nature at levels that exceed their aqueous solubility) in ameliorating the rarity of such data.

§1.5 Thesis Contents and Structure

This thesis composes of five chapters. Chapter One presents the introduction to the study including the general background, significance of study, justification of study, aims and objectives. Chapter Two reviews the relevant literatures related to the identities and properties of the investigated substances and also, previous studies on environmental contamination by these chemicals are reviewed, with a view to identify the research gaps that the present study sought to fill. In Chapter Three, the materials and methods (experimental procedures) employed to achieve research objectives are discussed. The results of all the experimental analyses as well as results of statistical analyses are presented and discussed in Chapter Four. Chapter Five includes a summary of the study results, conclusions arrived at and recommendations made.

CHAPTER TWO: LITERATURE REVIEWS

The phthalate esters investigated in this research are briefly introduced in this chapter. The reviews (i.e., surveys of articles, books, conference papers, theses, etc; or accounts of what has been published on a topic by accredited scholars and researchers) focused on the identities, physical and chemical properties, sources, environmental fates

as well as on public health, ecological effects, and contamination status and historical trends of these toxicants in the biotic and abiotic environments. Owing to the scarcity of literatures on the environmental contamination/pollution and/or PAEs biodegradation in People's Republic of China, literatures on other parts of the world highlights the gaps (or strengths and weaknesses) that this study sought to fill. This chapter also contains a brief review of previous researches on other related topics. It also reflects the problem's history and the today's situation, and describes the most popular and respected points of view on the problem.

§2.1 General Background to Phthalic Acidic Esters (PAEs)

2.1.1 History

The development of cellulose nitrate in 1846 led to castor oil being patented in 1856 for use as the first plasticizer. In 1870 camphor became the more favored plasticizer for cellulose nitrate. Phthalates were first introduced in the 1920s and quickly replaced the volatile and odorous camphor. In 1931 the commercial availability of polyvinyl chloride and the development of di-2-ethylhexyl phthalate began the boom of the plasticizer PVC industry.

2.1.2 The basic chemical structure of Phthalates

The basic chemical structure of phthalates is benzene dicarboxylic acid with two side chains, which can be alkyl, benzyl, phenyl, cycloalkyl, or alkoxy groups (Fig. 2.1). General chemical structure of phthalates is shown in Fig. 2.1 (a), where R and $R' = C_nH_{2n+1}$; $n = 4-15$. When R and R' are the same, then the phthalate is a di-ester. Studies have demonstrated that phthalates with shorter ester chains like dimethyl phthalate (DMP), diethyl phthalate [DEP; Fig. 2.1 (b)], dibutyl phthalate [DBP; Fig. 2.1 (c)], diphenyl phthalate (DPP), dipropyl phthalate (DPrP), and butyl-benzyl phthalate (BBP) can be readily biodegraded and mineralized. On the other hand, phthalates with longer ester chains, such as dicyclohexyl phthalate (DCP), dihexyl phthalate (DHP), dioctyl phthalate (DOP), and di-2-ethylhexyl phthalate (DEHP) are less susceptible to biodegradation (Wang *et al.* 2000; Chang *et al.* 2004).

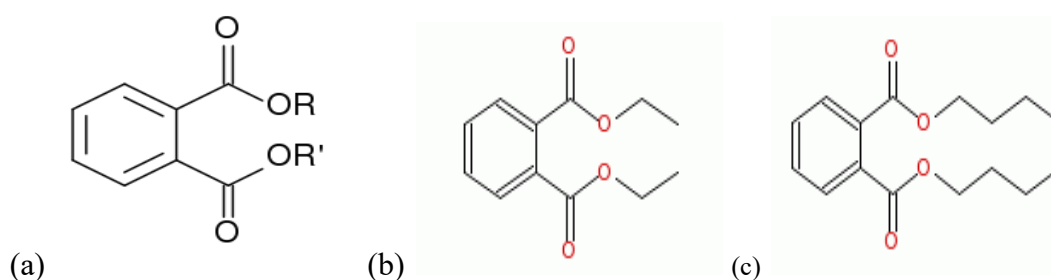


Fig. 2.1. (a) General chemical structure of phthalates; and basic chemical structures of (b) diethyl phthalate (DEP) and (c) dibutyl phthalate (DBP)

2.1.3 Uses of phthalates

Phthalates are used in a large variety of products, from enteric coatings of pharmaceutical pills and nutritional supplements to viscosity control agents, gelling agents, film formers, stabilizers, dispersants, lubricants, binders, emulsifying agents, and suspending agents. End applications include adhesives and glues, agricultural adjuvants, building materials, personal care products, medical devices, detergents and surfactants, packaging, children's

toys, modeling clay, waxes, paints, printing inks and coatings, pharmaceuticals, food products, and textiles. Phthalates are also frequently used in soft plastic fishing lures, caulk, paint pigments, and sex toys made of so-called “jelly rubber”. Phthalates are used in a variety of household applications such as shower curtains, vinyl upholstery, adhesives, floor tiles, food containers and wrappers, and cleaning materials. Personal care items containing phthalates include perfume, eye shadow, moisturizer, nail polish, liquid soap, and hair spray (Rudel and Perovic, 2008). They are also found in modern electronics and medical applications such as catheters and blood transfusion devices. The most widely-used phthalates are the di-2-ethyl hexyl phthalate (DEHP), the diisodecyl phthalate (DIDP) and the diisononyl phthalate (DINP). DEHP is the dominant plasticizer used in PVC due to its low cost. Benzylbutylphthalate (BBP) is used in the manufacture of foamed PVC, which is mostly used as a flooring material. Phthalates with small R and R' groups (see Table 2.1) are used as solvents in perfumes and pesticides.

The largest usage of DBP in general is as a plasticizer in resins and polymers such as polyvinyl chloride. Plasticizers are materials incorporated into a plastic in order to increase its workability and distendability. DBP is further used in printing inks, adhesives, alants/grouting agents, nitrocellulose paints, film coatings and glass fibres. The ubiquity of DBP in consumer products is demonstrated by its wide usage in cosmetics: a perfume solvent and fixative, a suspension agent for solids in aerosols, a lubricant for aerosol valves, an antifoamer, a skin emollient and a plasticizer in nail polish and fingernail elongators. Based on 1997 data, on average around 76% of DBP is used as a plasticizer in polymers, 14% in adhesives, 7% in printing inks and the remaining 3% of DBP is used in miscellaneous other applications (EU, 1999).

Table 2.1. Table showing the most commonly found phthalates

Name	Acronym	Structural Formula	*CAS no.
Dimethyl phthalate	DMP	$C_6H_4(COOCH_3)_2$	131-11-3
Diethyl phthalate	DEP	$C_6H_4(COOC_2H_5)_2$	84-66-2
Diallyl phthalate	DAP	$C_6H_4(COOCH_2CH=CH_2)_2$	131-17-9
Dipropyl phthalate	DPP	$C_6H_4[COO(CH_2)_2CH_3]_2$	131-16-8
Dibutyl phthalate	DBP	$C_6H_4[COO(CH_2)_3CH_3]_2$	84-74-2

* CAS = *Chemical Abstracts Services*

2.1.4 Production

As of 2004 manufacturers produced about 363 thousand metric tonnes (800 million pounds or 400 000 short tons) of phthalates each year. They contribute 10-60% of plastic products by weight (Rudel and Perovic, 2008). DBP is produced by the reaction of phthalic anhydride with n-butanol in the presence of concentrated sulphuric acid as a catalyst. Excess alcohol is recovered and recycled and the DBP is purified by vacuum distillation and/or activated charcoal. In 1998 the production volume of dibutyl phthalate (DBP) in the EU was estimated at 26,000

tonnes, of which 8,000 tonnes was thought to be exported outside the EU (EU, 1999), leading to a use volume of about 18,000 tonnes per annum (t a^{-1}). There is no import of DBP from outside the EU and there is also a clear decreasing trend in the production of DBP: 49,000 t a^{-1} (1994) – 37,000 t a^{-1} (1997) – 26,000 t a^{-1} (1998). The production ($>1,000$ tonnes) of DBP in 1998 was located at three production sites in the EU.

2.1.5 Properties

Phthalate esters are the dialkyl or alkyl aryl esters of phthalic acid (also called 1,2-benzenedicarboxylic acid, not be confused with the isomeric terephthalic or isophthalic acids); the name *phthalate* derives from phthalic acid, which itself is derived from word “naphthalene”. When added to plastics, phthalates allow the long polyvinyl molecules to slide against one another. The phthalates have a clear syrupy liquid consistency and show low water solubility, high oil solubility, and low volatility (Table 2.2). The polar carboxyl group contributes little to the physical properties of the phthalates, except when R and R' are very small (such as ethyl or methyl groups) (Liang, 2008). They are colorless, odorless liquids (Table 2.2) produced by reacting phthalic anhydride with an appropriate alcohol (usually 6 to 13 carbons).

2.1.6 Distribution and Bioaccumulation

The Henry's Law constants of $8.83\text{E}^{-7} \text{ atm.m}^3 \text{ mol}^{-1}$ and $2.66\text{E}^{-7} \text{ atm.m}^3 \text{ mol}^{-1}$ respectively indicate that DEP and DBP will only slowly volatilize from surface waters, i.e. virtually all of the DEP and DBP will remain in the water phase at equilibrium.

The octanol/water partition coefficients (K_{ow}) of DEP and DBP are high and consequently the equilibrium between water and organic carbon in soil or sediment will be very much in favor of the soil or sediment. Despite their low volatility, DEP and DBP have been reported as particulates and as vapors in the atmosphere. In the air DBP is transported and removed by both wet and dry deposition.

The high K_{ow} values of DEP and DBP indicate that the compounds have a potential for bioaccumulation. However, the actual degree of bioaccumulation *in vivo* will be determined by the metabolization and the elimination rate of the substance.

2.1.7 Health effects

2.1.7.1 Exposure

Phthalates are easily released into the environment because there is no covalent bond between the phthalates and plastics in which they are mixed. PAEs may be released into the environment during its production and subsequent life cycle stages, including disposal. As plastics age and break down, the release of phthalates accelerates. Phthalates in the environment are subject to biodegradation, photodegradation, and anaerobic degradation and, therefore, they do not generally persist in the outdoor environment. Outdoor air concentrations are higher in urban and suburban areas than in rural and remote areas (Rudel and Perovic, 2008). Emissions to water and air are expected to be the most important entry routes of PAEs.

Indoor air concentrations are generally higher than outdoor air concentrations due to the nature of the sources. Because of their volatility, DEP and DMP are present in higher concentrations in air in comparison with the heavier and less volatile DEHP. Higher air temperatures result in higher concentrations of phthalates in the air. PVC flooring leads to higher concentrations of BBP and DEHP which are more prevalent in dust (Rudel and Perovic, 2008).

Table 2.2. Selected properties of DEP and DBP (Adapted from Staples et al., 1997)

Properties	DEP	DBP
Identification of the compounds		
CAS No	84-66-2	84-74-2
EINECS No	201-550-6	201-557-4
IUPAC name	diethyl benzene-1,2-dicarboxylate	dibutyl benzene-1,2-dicarboxylate
Trade name	DEP	DBP
Molecular weight	222.24	278.34
Molecular formula	C ₁₂ H ₁₄ O ₄	C ₁₆ H ₂₂ O ₄
Physico-chemical properties		
Purity	>99% (w/w)	>99% (w/w)
Physical state	colorless, oily liquid, slightly aromatic odor	colorless, oily liquid, slightly aromatic odor
Melting point	- 35 °C	- 40 °C
Boiling point	293 °C at 1,013 hPa	340 °C at 1,013 hPa
Relative density	1.118 g cm ⁻³ at 20°C	1.042 g cm ⁻³ at 20°C
Vapour pressure (mm Hg)	1.0E ⁻³ at 25°C	2.7E ⁻⁵ at 25°C
Water solubility	1100 mg l ⁻¹ at 20°C	11.2 mg l ⁻¹ at 20°C
n-octanol/water (log K _{ow})	2.38	4.45
Specific gravity at 20 °C	1.118	1.042
ACD logP	2.7	4.825
Washout ratio (W)	9.0E ⁺⁴	2.8E ⁺⁴
Granulometry	not applicable	not applicable
Flammability	negative	Negative
Explosive properties:	negative	Negative
Oxidizing properties	negative	Negative

People are commonly exposed to phthalates, and most Americans tested by the Centers for Disease Control and Prevention (CDCP) have metabolites of multiple phthalates in their urine. Because phthalate plasticizers are not chemically bound to PVC they can easily leach and evaporate into food or the atmosphere. Phthalate exposure can be through direct use or indirectly through leaching and general environmental contamination. Diet is believed to be the main source of DEHP and other phthalates in the general population. Fatty foods such as milk, butter, and meats are a major source. Low molecular weight phthalates such as DEP, DBP, Butylbenzyl phthalate

(BBzP) may be dermally absorbed. Inhalational exposure is also significant with the more volatile phthalates (Heudorf *et al.*, 2007).

In a 2008 Bulgarian study higher dust concentrations of DEHP were found in homes of children with asthma and allergies, compared with healthy children's homes (Kolarik *et al.*, 2008). The author of the study stated, “The concentration of DEHP was found to be significantly associated with wheezing in the last 12 months as reported by the parents” (Kolarik *et al.*, 2008). Phthalates were found in almost every sampled home in Bulgaria. The same study found that DEHP, BBzP, and DnOP were in significantly higher concentrations in dust samples collected in homes where polishing agents were used. Data on flooring materials was collected but there was not a significant difference in concentrations between homes where no polish was used that have balatum (PVC or linoleum) flooring verses homes with wood. High frequency of dusting did decrease the concentration (Kolarik *et al.*, 2008).

Children's exposure to phthalates generally is greater than adults. In a 1990's Canadian study that modeled ambient exposures it was estimated that daily exposure to DEHP was 9 microgram per kilogram (mcg kg^{-1}) bodyweight day^{-1} in infants, 19 mcg kg^{-1} bodyweight day^{-1} in toddlers, 14 mcg kg^{-1} bodyweight day^{-1} in children, and 6 mcg kg^{-1} bodyweight day^{-1} in adults (Heudorf *et al.*, 2007). Infants and toddlers are at the greatest risk of exposure due to their mouthing behavior. Body care products containing phthalates are a source of exposure for infants. The authors of a 2008 study “observed that reported use of infant lotion, infant powder, and infant shampoo were associated with increased infant urine concentrations of phthalate metabolites, and this association is strongest in younger infants. These findings suggest that dermal exposures may contribute significantly to phthalate body burden in this population”. Though they did not examine health outcomes, they noted that “Young infants are more vulnerable to the potential adverse effects of phthalates given their increased dosage per unit body surface area, metabolic capabilities, and developing endocrine and reproductive systems” (Sathyanarayana *et al.*, 2008).

In 2008 the Danish Environmental Protection Agency (EPA) found a variety of phthalates in erasers and warned of health risks when children regularly suck and chew on them. The European Commission Scientific Committee on Health and Environmental Risks (SCHER), however, considers that, even in the case when children bite off pieces from erasers and swallow them, it is unlikely that this exposure leads to health consequences (GreenFacts, 2009).

Phthalates are also found in medications, where they are used as inactive ingredients in producing enteric coatings. It's not known how many medications are made using phthalates, but some include omeprazole, didanosine, mesalamine, and theophylline. A recent study found that urinary concentrations of monobutyl phthalate, the DBP metabolite, of mesalamine users was 50 times higher than the mean of nonusers (some formulations of mesalamine do not contain phthalates) (Hernández-Díaz *et al.*, 2009). The study showed that exposures from phthalate containing medications can far exceed population levels from other sources

(Hernández-Díaz *et al.*, 2009). DBP in medications raises concern about health risks due to the high level of exposures associated with taking these medications especially in vulnerable segments of the population, including pregnant women and children (Hernández-Díaz *et al.*, 2009).

In 2008 the US National Research Council (USNR) recommended that the cumulative effects of phthalates and other antiandrogens be investigated. It criticized US EPA guidances, which stipulate that when examining cumulative effects the chemicals examined should have similar mechanisms of action or similar structures, as too restrictive. It recommended instead that the effects of chemicals which cause similar adverse outcomes should be examined cumulatively (Hernández-Díaz *et al.*, 2009). Thus the effect of phthalates should be examined together with other antiandrogens, which otherwise may have been excluded because their mechanisms or structure were different. In a single study of girls with precocious puberty, the authors reported no difference in phthalate levels in girls with or without the condition, leading them to conclude that phthalates were not a cause of precocious puberty in humans (Lomenick *et al.*, 2009).

2.1.7.2 Endocrine disruption

In studies of rodents exposed to certain phthalates high doses have been shown to change hormone levels and cause birth defects (USCDC, 2005). A recent British study showed that the phthalate dibutyl phthalate (DBP) or its metabolite monobutyl phthalate (MBP) suppresses steroidogenesis by fetal-type Leydig cells in primates as in rodents (Hallmark *et al.*, 2007).

A study published in 2005, lead investigator Dr. Shanna Swan (hence also called the “Swan Study”) reported that human phthalate exposure during pregnancy resulted in decreased anogenital distance among baby boys. In this study phthalate metabolites were measured in urine samples collected from the pregnant women who gave birth to the infants. After birth the genital features and anogenital distance of these women's babies were measured and correlated with the residue levels in the mother's urine. Boys born to mothers with the highest levels of phthalates were 7 times more likely to have a shortened anogenital distance (Swan *et al.*, 2005). An editorial concerning this paper in the same volume stated that the study population was small, and “needs to be investigated more thoroughly in a larger, more diverse population” (Barrett, 2005). While anogenital distance is routinely used as a measure of fetal exposure to endocrine disruptors in animals (Salazar-Martinez *et al.*, 2004), this parameter is rarely assessed in humans and its significance is unknown (Postellon, 2008). One paper states that “Whether anogenital distance measurements in humans relate to clinically important outcomes ... remains to be determined,” (Romano-Riquer *et al.*, 2007) and a National Toxicology Program expert panel concluded that anogenital distance is a “‘novel index’ whose relevance in humans ‘has not been established,’” and that there is “insufficient evidence in humans” that DEHP causes harm (Kaiser, 2005). The Swan study is thought by some to “suggest that male reproductive development in humans could be affected by prenatal exposure to environmentally relevant levels of phthalates” (Tilson, 2008). Authors of a more recent study of boys with undescended testis hypothesized that exposure to a combination of phthalates and anti-androgenic pesticides may

have contributed to that condition (Toppari *et al.*, 2006).

In contrast to the Swan study an earlier study found that “adolescents exposed to significant quantities of DEHP as neonates showed no significant adverse effects on their physical growth and pubertal maturity” (Rais Bahrami *et al.*, 2004). This study, however, examined children exposed intravenously to phthalate diesters, and intravenous exposure results in little metabolic conversion of the relatively nontoxic phthalate diester to its more toxic monoester metabolite (Huber *et al.*, 1996).

In November 2009, Swan *et al.*, in the *International Journal of Andrology*, in a paper titled “*Prenatal phthalate exposure and reduced masculine play in boys*”,

“... suggest that prenatal exposure to antiandrogenic phthalates may be associated with less male-typical play behaviour in boys. ... [and] ... suggest that these ubiquitous environmental chemicals have the potential to alter androgen-responsive brain development in humans” (Swan *et al.*, 1999).

2.1.7.3 Other effects

There may be link between the obesity epidemic and endocrine disruption and metabolic interference. Studies conducted on mice exposed to phthalates in utero did not result in metabolic disorder in adults (Desvergne *et al.*, 2009). Although, “in a national cross-section of U.S. men, concentrations of several prevalent phthalate metabolites showed statistically significant correlations with abnormal obesity and insulin resistance” (Desvergne *et al.*, 2009). Mono-ethyl-hexyl-phthalate, a metabolite of DEHP, has been found to interact with all three peroxisome proliferator-activated receptors (PPARs) (Desvergne *et al.*, 2009). PPARs are members of the nuclear receptor superfamily. The author of the study stated “The roles of PPARs in lipid and carbohydrate metabolism raise the question of their activation by a sub-class of pollutants, tentatively named metabolic disruptors” (Desvergne *et al.*, 2009). Phthalates belong to this class of metabolic disruptors. It is a possibility that over many years of exposure to these metabolic disruptors, they are able to deregulate complex metabolic pathways in a subtle manner (Desvergne *et al.*, 2009).

Large amounts of specific phthalates fed to rodents have been shown to damage their liver and testes (USCDC, 2005), and initial rodent studies also indicated hepatocarcinogenicity. Following this result diethyl hexyl phthalate was listed as a possible carcinogen by IARC, EC, and WHO. Later studies on primates showed that the mechanism was specific to rodents - humans are resistant to the effect (Consumer Product Safety Commission, CPSC, 2001). The carcinogen classification was subsequently withdrawn.

In 2004 a joint Swedish-Danish epidemiologic team found a link between allergies in children and the phthalates DEHP and BBzP. Their review article and meta-analysis of published data relating to phthalates and asthma found an association between phthalates in the home and asthma, especially in children, but this evidence was limited by imprecise data on levels of exposure (Bornehag *et al.*, 2004).

In 2007 a cross-sectional study of U.S. males concluded that urine concentrations of four phthalate metabolites correlate with waist size and three phthalate metabolites correlate with the cellular resistance to insulin, a precursor to Type II diabetes. The authors note the need for follow-up longitudinal studies, as waist size is known to correlate with insulin resistance (Stahlhut *et al.*, 2007).

On November 15, 2009, South Korean scientists reported findings of a statistically-significant correlation between urine phthalate concentrations in children and symptoms of ADHD. Although more research is needed in order to conclusively determine the relationship between phthalate and ADHD, the article suggests that consumers should be aware of its potential effects on behavior and neurological disorders (Kim *et al.*, 2009). The findings were replicated in The Mount Sinai Children's Environmental Health Study, which enrolled a multiethnic prenatal population in New York City between 1998 and 2002 (n = 404), published in Jan 2010. There was an association of prenatal phthalate exposure with offspring behavior and executive functioning at ages 4 to 9 years (Engel *et al.*, 2010).

2.1.8 Legal status

2.1.8.1 European Union (EU)

The use of some phthalates has been restricted in the EU for use in children's toys since 1999 (EU, 1999). DEHP, BBP, and DBP are restricted for all toys; DINP, DIDP, and DNOP are restricted only in toys that can be taken into the mouth. The restriction states that the amount of phthalates may not be greater than 0.1% mass percent of the plasticized part of the toy. These phthalates are allowed at any concentration in other products and other phthalates are not restricted.

There are no other specific restrictions in the European Union although draft proposals have been tabled for the inclusion of BBP, DEHP and DBP on the Candidate list of Substances for Authorization under REACH (EU, 1999). The Dutch office of Greenpeace UK sought to encourage the EU to ban sex toys that contained phthalates (EU, 1999).

2.1.8.2 United States

In August 2008, the US Congress passed and President George W. Bush signed the Consumer Product Safety Improvement Act (CPSIA) which became public law 110-314 (GovTrack.us, 2008). Section 108 of that law specified that as of 10 February 2009, “it shall be unlawful for any person to manufacture for sale, offer for sale, distribute in commerce, or import into the United States any children’s toy or child care article that contains concentrations of more than 0.1 percent of” DEHP, DBP, or BBP and “it shall be unlawful for any person to manufacture for sale, offer for sale, distribute in commerce, or import into the United States any children’s toy that can be placed in a child’s mouth or child care article that contains concentrations of more than 0.1 percent of” DINP, DIDP, DnOP. Furthermore, the law requires the establishment of a permanent review board to

determine the safety of other phthalates. Prior to this legislation, the Consumer Product Safety Commission had determined that voluntary withdrawals of DEHP and DINP from teething rings, pacifiers, and rattles had eliminated the risk to children, and advised against enacting a phthalate ban (GovTrack.us, 2008). Some phthalates were restricted in children's toys sold in California starting in 2009 (Hileman, 2007).

2.1.8.3 Australia

In January 2010 the Australian Consumer Affairs Minister Craig Emerson announced a ban on items containing more than one per cent diethylhexyl phthalate (DEHP) because of international research linking it to reproductive difficulties (Emerson, 2010).

2.1.9 Identification in plastics

Phthalates are used in some but not all PVC formulations, and there are no labeling requirements for phthalates specifically. PVC plastics are typically used for various containers and hard packaging, medical tubing and bags, and are labeled “Type 3” for recycling reasons. However, the presence of phthalates rather than other plasticizers is not marked on PVC items. Plasticizers such as phthalates are only used for making flexible PVC, so it stands to reason that they should only be present in soft forms of PVC. If a more accurate test is needed, chemical analysis, for example by gas chromatography, can establish the presence of phthalates.

Polyethylene terephthalate ethylene (PETE) is the main substance used to package bottled water and many sodas. Products containing PETE are labeled “Type 1” (with a “1” in the recycle triangle) for recycling purposes. Although the word “phthalate” appears in the name, however, PETE is not a phthalate. They are chemically different substances (Rudel and Petrovich, 2008).

§2.2 Ecotoxicology and Environmental Chemistry of PAEs

2.2.1 Assessment of PAEs Exposure

2.2.1.1 Occupational exposure

Workplace exposure to PAEs is possible due to the production of PAEs, the production of products that contain PAEs and the use of those products. Occupational exposure levels have been estimated by European Union Risk Assessment Report (2003) using measured data and modelling by the “Estimation and Assessment of Substance Exposure” (EASE) with relevant assumptions.

Production of PAEs is done in closed systems. Exposure is mainly due to activities such as filling of tankers and drums, sampling, changing of filters and other maintenance activities. Typical full-shift inhalation exposure levels in production are estimated to be below 2 mg m^{-3} with a reasonable worst case of 5 mg m^{-3} . Short-term inhalation exposure levels of up to 10 mg m^{-3} are considered possible. Dermal exposure in production is expected to be highest for drumming of PAEs and was estimated by EASE to be up to 420 mg day^{-1} .

The formulation of products containing up to 15% of PAEs leads to inhalation exposure and dermal exposure mainly due to adding of the substance to mixers, mixing and forming of the products by processes such as extruding and calendaring. Mixing and forming processes are done at elevated temperatures (150-210°C). The estimated reasonable worst-case full-shift inhalation exposure level is 5 mg m⁻³ (typical: < 2 mg m⁻³, short-term 10 mg m⁻³). Manual addition of DEP or DBP is estimated to lead to a dermal exposure level of 420 mg day⁻¹.

The end use of products containing the substances occurs in the polymer industry, the painting industry and the printing industry and can be divided into two subscenarios: aerosol forming techniques, such as spray application, and techniques that do not involve aerosols. Inhalation exposure to PAEs in techniques that do not involve aerosols (e.g. application of a product by means of a brush) is estimated to be negligible. The reasonable worst-case full-shift inhalation exposure level is estimated to be 10 mg m⁻³ with typical values of 2 mg m⁻³ and short-term exposure levels of up to 20 mg m⁻³. Dermal exposure during prolonged spray application of products containing PAEs is estimated to be up to 975 mg day⁻¹. Other activities with products containing PAEs are expected to lead to lower dermal exposure levels.

2.2.1.2 Consumer exposure

PAEs are used in several consumer products. To cover the widespread use of DEP or DBP, European Union Risk Assessment Report (2003) focussed attention on products containing a relatively large concentration of DEP or DBP such as cosmetics (especially nail polish and enamels), adhesives and regenerated cellulose film (cellophane) wrapped food. Attention was also given to the (un)intentional use of DBP in children's toys, in view of the general public concern on the use of phthalates in PVC toys.

2.2.1.3 Human exposure via the environment

PAEs may be released to the environment through wastewater effluents and air at the sites where they are produced, formulated and/or processed/used. The European Union Risk Assessment Report (2003) estimated concentrations in the air near the emission sources for the various exposure scenarios ranging from 0.02 to 2.4 µg m⁻³. The calculated total daily human intake via air, drinking water and food for all emission scenarios at local scale (using "European Union System for the Evaluation of Substances", EUSES) ranged from 7.86 x 10⁻⁴ to 0.0925 milligram per kilogram body-weight per day (mg kg⁻¹ bw day⁻¹) for the various exposure scenarios. For the regional scale (EU), the concentration in the air and the total human intake are calculated to be 0.006 µg m⁻³ and 3.59 x 10⁻⁴ mg kg⁻¹ bw day⁻¹, respectively.

DBP has been identified in human breast milk in concentrations ranging from 10 to 51 µg kg⁻¹. Whether the DBP in human breast milk originates from direct or from indirect sources is not clear, but given the diffuse use and the diffuse emissions in the environment, the latter is more likely. Based on an average daily consumption of 120 g human milk kg⁻¹ bw for infants, the exposure via breast milk for infants varies between 1.2 and 6 µg DBP kg⁻¹

bw day⁻¹ (European Union Risk Assessment Report, 2003).

2.2.2 The biodegradability of phthalates

The biodegradability difference of phthalates is likely due to the steric effect of phthalates side ester chains, which hinders the hydrolytic enzymes from binding to the phthalates and thereby inhibits their hydrolysis. This has been supported by Xia *et al.* (2004) in a study of quantitative structure-activity relationship analysis of phthalates and their aerobic biodegradation. On the other hand, the branched ester chain is not a significant factor limiting the degradation of phthalates (Ejlertsson *et al.*, 1997).

Different phthalates or phthalate isomers show different biodegradation rates, and phthalate-hydrolyzing enzymes are structurally specific. Gu *et al.* (2005) reported that a dimethyl isophthalate (DMI)-hydrolyzing enzyme purified from *Rhodococcus erythropolis* readily hydrolyzed DMI and DMP while hardly hydrolyzed dimethyl terephthalate (DMT).

The contribution of hydrolysis to the overall environmental degradation of phthalate esters, including DBP, is expected to be low. Photo-oxidation by OH radicals contributes to the elimination of DEP and DBP from the atmosphere. The metabolic pathway of aerobic and anaerobic biodegradation of phthalates can be summarised as follows. **First**, the di-ester is hydrolysed into the mono-ester by esterases with low substrate specificity. **Subsequently**, the mono-ester is converted into phthalic acid. There is ample evidence that DEP and DBP are readily biodegradable under aerobic conditions (Staples *et al.*, 1997; European Union Risk Assessment Report, 2003; Liang, 2008; etc). The same literature sources indicate that biodegradation of DEP and DBP is much slower in the anaerobic environment, e.g. sediments or deeper soil or groundwater layers.

2.2.2.1 Phthalates Biodegradation by pure culture

Most phthalate-degrading isolates are either aerobes or facultative aerobes, showing that the phthalate-degrading bacteria are mainly from four divisions in *Bacteria*, i.e., *Proteobacteria*, *Actinobacteria* (or high G+C), *Firmicutes* (or low G+C), and *Bacterioids/Chlorobi*. Among them, the commonly found genera are *Sphingomonas* (α -*Proteobacteria*), *Comamonas* (β -*Proteobacteria*), *Pseudomonas* (γ -*Proteobacteria*), as well as *Arthrobacter* and *Rhodococcus*.

However, anaerobic phthalate-degrading isolates were little known because of the lack of syntrophic conditions. So far, only three isolates, one *Clostridium* sp. and two *Bacillus* sp. strains, were found to be solely capable of degrading phthalates anaerobically (Chang *et al.*, 2005a).

In addition to *Bacteria*, a few fungi species, including *Aspergillus niger* AG-1 (Ganji *et al.*, 1995), *Sclerotium rolfsii* (Sivamurthy *et al.*, 1991), *Penicillium lilacinum* (Engelhardt *et al.*, 1977), *Fusarium oxysporum* (Kim and Lee, 2005), *Phanerochaete chrysosporium*, *Trametes versicolor*, *Daldinia concentrica* (Lee *et al.*, 2004), and *Polyporus brumalis* (Lee *et al.*, 2007), as well as green microalga *Closterium lunula* (Yan and Pan, 2004) and *Chlorella pyrenoidosa* (Yan *et al.*, 2002), can also degrade phthalates. *The broad-range degradation ability of*

fungi is attributed to the strong extracellular ligninolytic enzymes, such as lignin peroxidase, manganese-dependent peroxidase, and laccase. For instance, the cutinase-producing *F. oxysporum* showed high enzymatic activities and was able to degrade BBP (Kim *et al.*, 2002), DEHP (Kim *et al.*, 2003), DBP (Kim and Lee, 2005), DPrP (Kim *et al.*, 2005), dipentyl phthalate (Ahn *et al.*, 2006), and DHP (Kim *et al.*, 2007) within several hours.

2.2.2.2 Phthalates biodegradation by mixed culture

In the natural environments, complete degradation of complex organics, like phthalates, is always carried out syntrophically by several members of organisms (Gu *et al.*, 2005). The biodegradation of phthalates primarily involves the sequential hydrolysis of ester linkage, which results in monoesters and, subsequently, PA, while forming alcohols simultaneously. Thus, the microbial assimilation of phthalates requires diverse metabolic genes and enzymes, indicating a single organism is unlikely able to completely mineralize phthalates (Staples *et al.*, 1997).

So far, mixed culture consortia have been shown to completely mineralize phthalates. Wang *et al.* (2004c) reported that DMP was completely mineralized by two consortia of microorganisms; one comprised of *Pseudomonas fluorescens*, *P. aureofaciens*, and *Sphingomonas paucimobilis*, whereas the other comprised of *Xanthomonas maltophilia* and *S. paucimobilis*. DMP was also found to be serially degraded by a coculture of an *Arthrobacter* sp. and *S. paucimobilis*. The former species was responsible for transforming DMP into MMP, which was utilized by the latter species (Vega and Bastide, 2003).

In addition, mixed cultures can also increase phthalates degradation rates. In the presence of both *Sphingomonas* sp. DK4 and *Corynebacterium* sp. O18, the degradation rates of the eight phthalates examined were enhanced (Chang *et al.*, 2004).

2.2.2.3 Biodegradations of phthalates under various environmental conditions

Phthalates biodegradation under various environmental conditions, such as in wastewater treatment processes, sludge, freshwater and sediment, soils, and landfill sites, have been extensively studied. Generally, phthalates biodegradation shares the following common characteristics in different environments: (1) a decrease in biodegradability with the increase in phthalates side chain length; (2) anaerobic degradation of phthalates is generally much slower than aerobic degradation (Staples *et al.*, 1997; Yuan *et al.*, 2002); (3) the primary degradation of phthalates is always expressed in the first-order kinetics (Wang *et al.*, 1997b; Gavala *et al.*, 2003); (4) high concentration of phthalates and/or their metabolites inhibit their biodegradation.

2.2.2.3.1 Wastewater treatment processes

Discharge from sewage works is a major source of phthalates into the environment. In comparison to other phthalates, DEP and DEPH, in the ranges of 1-74 and 28-122 $\mu\text{g L}^{-1}$, respectively, have been found in higher concentrations in raw sewage (Marttinen *et al.*, 2003a). Generally, phthalates are not readily mineralized in the

wastewater treatment processes since high hydrophobicity and low solubility allow phthalates to be adsorbed to suspended organic matter and subsequently transferred to settled sludges (Gavala *et al.*, 2003).

The fate of phthalates in wastewater treatment plant (WWTP) - and laboratory-scaled reactors, such as sequencing batch reactor, packed-bed reactor, and upflow anaerobic sludge blanket (UASB) reactor, has also been studied broadly. Although adsorption is found to be dominant, biodegradation also contributes to the removal of phthalates. Marttinen *et al.* (2003) studied the DEHP degradation in a WWTP using mass balance analysis and found that DEHP removal efficiency from the water phase was approximately 94%, 29% of which was attributed to biodegradation. A similar result was found in a trickle filter WWTP, where the biodegradation efficiency of DEHP was found to be in the range 1% to 44% (Oliver *et al.*, 2005). Fauser *et al.* (2003) studied the fate of phthalates in WWTP, and the results showed that with the mean inlet of 240 g-DEHP/day, 70% DEHP was biodegraded, and 28% was in the sludge (Fauser *et al.*, 2003). Roslev *et al.* (2007) reported a pronounced biodegradation of different phthalates in WWTP where the overall microbial degradation of DMP, DBP, BBP, and DEHP was estimated to be 93%, 91%, 90%, and 81%, respectively.

Phthalate degradation in reactors is greatly dependent upon the reactor design and seed sludge. For instance, compared to the *continuous flow* mode, the *recirculating flow* mode of a trickling filter microcosm improved the DEP and DEHP removal efficiency to 99% in the 48 h and 85% in 96 h, respectively (Oliver *et al.*, 2007). Complete removal of DMP in wastewater was accomplished using a packed-bed reactor with an acclimated mixed bacterial culture (Juneson *et al.*, 2002). Marttinen *et al.* (2004b) reported that the sequencing batch reactor could efficiently remove 95% of DEHP, together with $\text{NH}_4\text{-N}$, from reject water originating from sewage sludge. However, the type of inoculum used may play a very important role for quick degradation (Juneson *et al.*, 2001).

Anaerobic reactors were successful in the degradation of DMP (Liang *et al.*, 2007) and phthalate isomers, such as PA (Tur and Huang 1997) and TA (Kleerebezem *et al.*, 1997, 1999a; Wu *et al.*, 2001; Kleerebezem *et al.*, 2005), in wastewater. Liang *et al.*, (2007) reported that more than 99% of DMP (600 mg L^{-1}) and 93% of *chemical oxygen demand (COD)* were effectively removed in a UASB reactor at an 8-h hydraulic retention time. A full-scale TA wastewater treatment plant was able to remove 90% of COD and 85-90% of TA, with an organic loading rate of $3 \text{ g-COD (Lday)}^{-1}$ and 1 day of hydraulic retention time (Qiu *et al.*, 2004). In a two-stage UASB, TA was degraded at the rate of $15 \text{ g-COD (Lday)}^{-1}$ after 125 days of activation (Kleerebezem *et al.*, 2005). By introducing a bio-filter or reticulated polyurethane foam particles into a UASB, anaerobic hybrid reactors exhibited high removal capabilities in PA and TA.

2.2.2.3.2 Sludge

Sewage sludge contains relatively high concentrations of phthalates as a result of accumulation from the urban runoff, drainage, and domestic and industrial discharges. Most of the phthalates are biodegradable in sludge, and first-order kinetics is frequently used to describe their biodegradation. As mentioned above, phthalate

biodegradation under anaerobic conditions is much slower than under aerobic conditions, and the degradation rate is dependent on the inoculum. Kleerebezem *et al.* (1999b) reported that all three phthalate isomers and their corresponding dimethyl-esters could be completely mineralized anaerobically by all seed sludge studied, such as digested sludge and granular anaerobic sludge. However, the lag phases required for 50% of degradation of these compounds are usually quite long and may range from 17 to 156 days.

DEHP is a major pollutant identified at high concentrations in digested sludge, and its concentrations range from several to hundreds of mg kg^{-1} in dry weight (Cheng *et al.*, 2000). Although slow, DEHP removal in sludge under composting aerobic and anaerobic conditions has been verified. Marttinen *et al.* (2004a) reported that composting removed 58% and 34% of DEHP from raw sludge and digested sludge during 85 days stabilization. In another study, DEHP in sewage sludge was removed 94% by composting or by mixing with thermally dried soil within 9 months (Bago *et al.*, 2005). Under aerobic condition, the biodegradation of DEHP was 33-41% and 50-62% in 7 and 28 days in sludge, respectively (Marttinen *et al.*, 2004a). Anaerobically, DEHP degradation was in the range of 23-61% under thermophilic and mesophilic conditions (El-Hadj *et al.*, 2006).

High concentrations of DEHP (more than 60 mg L^{-1}) in digested sludge are likely to have a negative effect on degradation of other phthalates, such as DBP, DEP, and DMP, as well as the biogas production in sludge digesters (O'Connor *et al.*, 1989; Alatrisme-Mondragon *et al.*, 2003; Gavala *et al.*, 2003). Battersby and Wilson (1989) reported that increasing concentrations of DEHP (25, 50, 100, and 200 mg L^{-1}) decrease biogas from 48% to 6% of the theoretical gas production over a period of 60 days. This is probably caused by the partial metabolism of DEHP and the production of the intermediate 2-ethyl hexanol, which has been reported to be toxic to the methanogenic process (Ejlertsson and Svensson 1997). On the other hand, DEHP in sludge was found to degrade under good ventilation, sufficient sunlight, and proper moisture conditions (Cheng *et al.*, 2000). The half-lives of DEHP have been reported to be 45.4 and 28.9 days in composting lagoon sludge and activated sludge under aerobic conditions, respectively (Amir *et al.*, 2005).

2.2.2.3.3 Freshwater and sediment

Phthalates are ubiquitously detected in freshwater and tend to adsorb on sediment (Chang *et al.*, 2005a). Among eight phthalates found in Taiwan's rivers, DEHP was in the highest concentration ranging from less than 0.1 to $18.5 \text{ } \mu\text{g L}^{-1}$ in river water and 0.5 to $23.9 \text{ } \mu\text{g g}^{-1}$ in sediment (Yuan *et al.*, 2002), which is in the same range with many other places in the world (Turner and Rawling, 2000). These eight phthalates could be biodegraded in the river sediment with the average half-lives of 2.5-14.8 (aerobically) and 14.4-34.7 days (anaerobically). Generally, the biodegradation half-lives are ranged from less than 1 day to 2 weeks (Amir *et al.*, 2005). The biodegradation of DEHP ($52 \text{ } \mu\text{g L}^{-1}$) was almost complete in 2 days in water, and the biodegradation half-live was about 14 days (Yuwatini *et al.*, 2006).

Aerobic microorganisms, such as *Acinetobacter Iwoffii* (Hashizume *et al.*, 2002) and *Sphingomonas* sp. (Chang *et al.*, 2004; Kim *et al.*, 2008), and anaerobes, such as *Clostruium* sp. and *Bacillus* sp. (Chang *et al.*, 2005a), that can degrade phthalates have been isolated from river sediment recently. Although phthalate-degrading isolates are not as many as those from activated sludge and soil, these bacteria can potentially degrade phthalates in the freshwater environment. Additionally, the phytoplankton in the aquatic environment can also efficiently degrade phthalates under proper nitrogen and phosphorus nutrient and illumination conditions (Li *et al.*, 2007).

2.2.2.3.4 Soil and Slurry

Phthalate biodegradation in soil or soil slurry has been widely studied in the last decade and were mostly conducted using sludge-amended soil or the soils inoculated with phthalate-biodegrading enrichment (Wang *et al.*, 1997a; Roslev *et al.*, 1998; Madsen *et al.*, 1999; Cartwright *et al.*, 2000; Juneson *et al.*, 2001; Wang *et al.*, 2004a, b; Di Gennaro *et al.*, 2005; Zhou *et al.*, 2005; Chao *et al.*, 2006; Mohan *et al.*, 2006; Chao and Cheng, 2007; Shailaja *et al.*, 2007).

In a shaking flask test of phthalate biodegradation in sludge-amended soil, all of the 14 commercial phthalates as tested by Sugatt *et al.* (1984) were readily biodegraded, based on both primary and ultimate degradation criteria. Microorganisms in the sewage sludge were demonstrated to dominate DEHP degradation in sludge-amended soil (Roslev *et al.*, 1998; Madsen *et al.*, 1999). Similarly, DBP (100 $\mu\text{g g}^{-1}$) biodegradation in soil was reported to be enhanced by inoculation with DBP-degrading bacteria, although the indigenous microbial population in soil was capable of degrading DBP (Wang *et al.*, 1997a). The DEHP mineralization rate increased fourfold after inoculating the soil with DEHP-degrading bacteria SDE-2 (Roslev *et al.*, 1998). Additionally, three predominant strains, *B. iodinum*, *R. luteus*, and *B. brevis*, were isolated from DEHP-degrading contaminated soil. Inoculated with the DEHP-degrading bacteria enrichment into a soil-slurry sequential batch reactor (SBR), DEHP degradation half-time was only 2-5 days at ambient temperature (20-22 °C; Juneson *et al.*, 2001).

Mohan *et al.* (2006) and Shailaja *et al.* (2007) studied DEP and DBP biodegradation in soil, respectively, by using a slurry-phase reactor bioaugmented with the outlet from an effluent treatment plant. The degradation of DEP and DBP were almost completed within 48 and 72 h. The half-life for DEP in soil and aqueous phases were 1.19 and 2.52 days, respectively (Mohan *et al.*, 2006); the DBP degradation rate could reach 10.60 mg (L days)⁻¹ with a half-life of 0.75 day (Shailaja *et al.*, 2007). All these studies indicate that inoculating phthalate-degrading pure or mixed cultures enrichment into soil is a promising strategy for removing these hazardous compounds, which is also the basic idea of bioaugmentation. On the other hand, the treatment of contaminated soil in the slurry phase seems a good way for bioremediation, since slurry can improve the contact between microorganisms and contaminants by increasing the distribution of nutrients, terminal electron acceptors, and substrates.

2.2.2.3.5 Landfill Sites

The degradation intermediates of phthalates were detected in the majority of the landfill leachate (Jonsson *et al.*, 2003a, c), indicating phthalates were degraded in the landfill sites. Tests on BBP and DEHP in landfill reactors confirmed the phthalates degradation (Jonsson *et al.*, 2003b). In an anaerobic digester for municipal solid waste, DEP and PA (50-250 mg L⁻¹) could be completely degraded into methane and CO₂ (Ejlertsson *et al.*, 1996a). However, microorganisms in landfill environments have a substantially lower phthalate-degrading potential than those in the anaerobic reactors. Thus, waste sludge in the landfill site was suggested to enhance phthalates degradation (Ejlertsson *et al.*, 1996a).

Phthalates degradability was increased along with the aging process of the landfills (Ejlertsson *et al.*, 1996b). Phthalate diesters (PDEs) and the degradation products phthalate monoesters (PMEs) were below the detection limit in the acidogenic leachate from a full-scale young landfill site. During the transformation from acidic to initial methanogenic conditions, PDEs and PMEs concentrations decreased simultaneously (Ejlertsson *et al.*, 1996b; Jonsson *et al.*, 2003b). Sometimes, PMEs were present in concentrations several orders of magnitude higher than those of the PDEs (Jonsson *et al.*, 2003c). After the establishment of stable methanogenic conditions, PMEs further decreased. However, based on the studies of the nonenzymatic base-catalyzed hydrolysis of phthalates, Wolfe *et al.*, (1980) found that PMEs are more persistent than their corresponding PDEs. One hypothesis is that the carboxylate anion near the ester bond makes PMEs hard to hydrolyze, possibly due to the electrostatic repulsion toward the catalytic nucleophile attacking the ester bond (Maruyama *et al.*, 2005). To bind this anion and neutralized its negative charge, the PME esterase is proposed to have a positively charged amino acid, resulting the narrow substrate specificity of PME esterase.

2.2.2.3.6 Atmospheric compartment

There are a number of studies (Staples *et al.*, 1997; European Union Risk Assessment Report, 2003; Liang, 2008; etc) on the airborne toxicity of DEP and DBP to plants. In these studies, plants were exposed in a growth chamber or in a glasshouse to DEP or DBP vapor originating from plastics which contained DEP or DBP as a plasticizer or from substrates moistened with DEP or DBP. The results of the studies show a wide range of effect levels of DEP and DBP, ranging from 1.2 µg m⁻³ to 1,000 µg m⁻³. An average concentration of about 0.1 µg m⁻³ of each compound is considered to be a fairly good estimate of the plant.

2.2.2.4 The Biodegradation Pathways

The degradation pathways exploration of phthalates possibly will help to understand their mineralization process and the toxicological behavior of their metabolites (Nozawa and Maruyama, 1988; Horn *et al.*, 2004); and, in general, these pathways consist of two processes: *primary degradation* from phthalic di-esters (PDEs) to phthalate monoesters (PMEs) and then to phthalic acid (PA) and *ultimate biodegradation* from PA to carbon dioxide (CO₂) plus water (H₂O) and/or methane (CH₄) (Staples *et al.*, 1997).

2.2.2.4.1 Primary degradation pathway

Primary degradation consists of different type of pathways, including de-esterification or dealkylation, β -oxidation and *trans*-esterification.

(a) *De-esterification*: the most common one is the de-esterification of PDEs serially to form PME and PA. This degradation pathway is the same under both aerobic and anaerobic conditions (Shelton et al. 1984).

(b) *β -oxidation*: Phthalates with longer side chains than DEP are occasionally converted to those with shorter chains by β -oxidation, which removes one ethyl group each time (Amir *et al.*, 2005). Then, DEP is further converted to PA by two pathways, de-esterification and an alternative *trans*-esterification pathway.

(c) *Trans-esterification (or demethylation)*: DEP can be degraded by replacing an ethyl group with a methyl group in each step, producing ethyl-methyl phthalate (EMP) and dimethyl phthalate (DMP), the process of which is termed as *trans*-esterification (Cartwright *et al.*, 2000).

Few exceptions exist to the general pathways during phthalates biodegradation. For instance, in the biodegradation of DMP by *Arthrobacter* sp., one pathway was the hydrolysis of DMP directly into PA. In addition, DEP may also be directly degraded into PA without intermediates MEP by *Aureo-bacterium saperdae* NRRL B-14840 (Jackson *et al.*, 1996).

2.2.2.4.2 Ultimate degradation pathway

In the biodegradation of phthalates and polycyclic aromatic hydrocarbons [including phenanthrene (Kiyohara and Nagao, 1978), fluorine (Grifoll *et al.*, 1994) and fluoranthene] PA is a central intermediate (Eaton, 2001). Its pathways of ring cleavage are different under aerobic and anaerobic conditions (Cartwright *et al.*, 2000).

- Under the *aerobic condition*, PA is degraded by two dioxygenase-catalyzed pathways, forming the common intermediate protocatechuate, i.e. 3,4-dihydroxy benzoate (Eaton and Ribbons, 1982; Nomura *et al.*, 1992).

For **Gram-negative bacteria**, a dioxygenase catalyzes the formation of *cis*-4,5-dihydroxyphthalate, which is oxidized by an NAD-dependent dehydrogenase to form 4,5-dihydroxyphthalate. Protocatechuate is then formed by decarboxylation of 4,5-dihydroxyphthalate under the catalyzation of decarboxylases. For the **Gram-positive bacterium**, *Arthrobacter keyseri* (formerly *Micrococcus* sp.) 12B, PA is converted to protocatechuate through *cis*-3,4-dihydro-3,4-dihydroxyphthalate and 3,4-dihydroxyphthalate (Eaton and Ribbons, 1982).

Protocatechuate is metabolized further through either *ortho*- or *meta*-cleavage pathway by ring cleavage enzymes (Eaton and Ribbons, 1982). By the *meta*-cleavage pathway, protocatechuate is converted to 4-carboxy-2-hydroxymuconic semialdehyde, 2-hydroxy-4-carboxymuconic semialdehyde-hemiacetal, 4-oxalocitramalate, and then pyruvate and oxaloacetate. By the *ortho*-cleavage pathway, also known as the ketoadipate pathway, protocatechuate degrades into 3-ketoadipate. *P. fluorescens* and *P. putida* use the *ortho*-cleavage pathway (Dennis *et al.*, 1973), while *P. acidovorans* and *P. testosterone* use the *meta*-cleavage (Nakazawa and Hayashi,

1977). *A. keyseri* 12B used the *meta*-cleavage pathway (Keyser *et al.*, 1976), but its mutant strain 12C had the *ortho*-cleavage pathway (Eaton and Ribbons, 1982).

TA is readily used as a carbon source by aerobic bacteria (Keyser *et al.*, 1976). The pathway of TA under the aerobic condition is different from that of PA. TA is converted via benzoic acid, 4-hydroxybenzoate to protocatechuate (Naumova *et al.*, 1986), or via dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylic acid to protocatechuate (Schlafli *et al.*, 1994).

- *Anaerobic degradation* of PA was reported through decarboxylation to benzoate (Kleerebezem *et al.*, 1999b; Liu and Chi, 2003). Benzoate is then cleaved and degraded via β -oxidation, to hydrogen, carbon dioxide, and acetate (Elder and Kelly, 1994). Usually, the ***rate-determining step*** in anaerobic phthalates' degradation is from PA to benzoate (Kleerebezem *et al.*, 1999c).

§2.3 Related Reviews on Instrumentations

2.3.1 Microcalorimetry

The applications and interest in thermal analysis and calorimetry have grown enormously during the last half of the 20th century. The renaissance in these methods has been fueled by several influences, not least of which has been the revolution in instrumentation resulting from computer automation. The ingenuity and vision of analysts have also expanded to recognize the tremendous versatility of these techniques. They have long been used to characterize materials, decompositions, and transitions. We now appreciate the fact that these techniques have become indispensable or greatly increased their utility in studying many processes such as catalysis, hazards evaluation, etc., or/and in measuring important physical properties quickly, conveniently, and with markedly improved accuracy. Consequently, thermal analysis and calorimetry have grown in stature and more scientists and engineers have become, at least part time, practitioners.

The TAM III (third generation multi-channelled thermal activity monitor; Thermometric AB, Järfälla, Sweden) microcalorimeter in the isothermal mode, was employed to generate the thermogenic values/curves from the metabolism of each PAE by the three microbes *in situ* by continuously measuring the heat production rate (which is directly in relation to an increase or decrease in the energy release by a variety of sources), from the microbial respiration or thermal activities of the wide variety of processes taking place in the test sample, in terms of heat, heat flow and heat capacity over a temperature range of 15-150°C.

This modular tool, comprising thermostats, calorimeters, sample handling systems and auxiliary equipment, has three operating modes: isothermal mode, step-isothermal and scanning mode. Its performance and the details of its construction have been described in the User's Manual. The microcalorimetric technique proved to be a sound, efficient and useful tool, which requires only the knowledge of the initial and final energetic states and it is also independent of the organisms and reaction pathways. Temporary details (such as thermal effect, etc) not observable by other techniques and other much useful information, both qualitative and quantitative (like microbial growth rate constant, heat yield of microbial growth, etc), can also be revealed by microcalorimetric

method. Metabolic activities of living organisms result in a final exothermic effect in nature (Prado and Airoidi, 1999). In this respect, microcalorimetry is one of the techniques that can be applied to study metabolic activities of microorganisms (Zheng *et al.*, 2006; Ma *et al.*, 2007). Hence, this analytical method has been confirmed to be valid as an alternative in the investigation of substrate metabolism and microbial growth. Wide ranging practical applications of such a technique have been envisioned (Wadsö, 1980).

Microbial growth thermodynamics study encompasses an interesting combination of biology, chemistry and physics and the putting together of the numerous ideas from these individual disciplines. The use of microcalorimetry as a complementary method to study regulation and control of metabolic fluxes has great potential, since microcalorimetry with a high precision measures an average of all metabolic fluxes rather than the time dependence of the concentration of specific metabolic intermediates. It is therefore possible to use microcalorimetry as an accurate "fluxmeter" for studies of metabolic dynamics.

Information on the kinetics and thermodynamics of the reaction(s) investigated could be obtained by analyzing the data from the microcalorimetric power-time (P-T) curves (output from the heat conduction, isothermal microcalorimeter). Though molecular interpretations cannot be done from thermodynamic data, mechanistic investigations can be done on the basis of kinetic data. Several interested systems of reactions and processes are complex, and the analyses of data from such systems usually pose substantial difficulties. Microcalorimetry, as per its general nature, may provide some advantages for the investigations of such complex reactions, with no need for further investigations using other analytical tool.

The rate of heat production (dQ/dt or P) is correlated with microbial catabolic activity, such as the rate of O_2 consumption or the rate of CO_2 production, and overall microbial metabolic activity including both catabolism and anabolism, such as biomass growth according to the literatures of fairly recent studies (Cooney *et al.*, 1968; Luong and Volesky, 1980; Sparling, 1983; Yamano and Takahashi, 1983; Kimura and Takahashi, 1985; Lovrien *et al.*, 1989). Calorimetric (measuring a large quantity of heat) and microcalorimetric (measuring a very small quantity of heat, down to the microwatt, μW , level for dQ/dt or P) techniques can serve as an indirect measurement of a specific bioactivity that is not easily quantifiable in complex biosystems and bioprocesses, hence, the practical usefulness of these correlations. Moreover, as a consequence of their high degree of sensitivity, precision and accuracy, microcalorimetric measurements have been used as diagnostic and analytical tools in detecting and quantifying the effect of toxic compounds on the microbial metabolic activity (Kawabata *et al.*, 1983), in locating the end point of a particular reaction of the metabolic pathway (Ishikawa *et al.*, 1981), and in identifying the genetically modified cells of microorganisms.

2.3.2 Chromatographic Techniques

Chromatography is a word used to encompass a range of techniques in which mixtures of pure substances are separated into the individual substances by using a mobile phase (usually a liquid or gas) to push the mixture

along a stationary phase (usually a solid or liquid coated on a solid). Because the individual substances have different molecular structures, they interact differently with both the stationary and mobile phases, and consequently are "pushed" at different rates by the mobile phase. A number of chromatographic techniques are summarized in Table 2.3.

2.3.2.1 Gas Chromatography

Gas Chromatography (GC) is used to separate volatile components of a mixture. A micro amount of a sample to be analyzed is drawn up into a syringe. The syringe needle is placed into a hot injector port of the gas chromatograph, and the sample is injected. The injector is set to a temperature higher than the components' boiling points. So, components of the mixture evaporate into the gas phase inside the injector. A carrier gas, such as helium (He) or nitrogen (N₂), flows through the injector and pushes the gaseous components of the sample onto the GC column. It is within the column that separation of the components takes place. Molecules partition between the carrier gas (the mobile phase) and the high boiling liquid (the stationary phase) within the GC column. Two columns fit inside the oven of all GCs. A heating element is used to raise the oven temperature, when desired, and thus raise the column temperature. GC columns typically have a metal identification tag clipped onto the column that lists *column length* and *diameter*, *what material is inside*, and *the maximum operating temperature*. After components of the mixture move through the GC column, they reach a detector. Ideally, components of the mixture will reach the detector at varying times due to differences in the partitioning between mobile and stationary phases. The detector sends a signal to the chart recorder which results in a peak on the chart paper. *The area of the peak is proportional to the number of molecules generating the signal*

Table 2.3: Types of Chromatographic techniques used in this research

Type	Stationary phase	Mobile phase
Gas chromatography (GC)	Polar or non-polar liquid	Helium/N ₂ gas
Thin Layer Chromatography (TLC)	Solid on glass or plastic plate	Liquid

2.3.2.1.1 Simple steps to follow when using the GC:

- Wash a syringe 2-3 times with acetone* by filling the syringe completely and ejecting the waste acetone onto a paper towel.
- Pull some of the sample into the syringe*. One will most likely need to remove air bubbles in the syringe by

rapidly moving the plunger up and down while the needle is in the sample. Usually 1-2 μL of sample is injected into the GC. It is acceptable to have small air bubbles in the syringe. However, one does not want to inject mostly air or the resulting peaks will be too small on the chart recorder.

(c) *Make sure the chart recorder is on and set to the appropriate chart speed.* Set the baseline using the zero on the chart recorder. With the pen in place, turn on the chart, make sure the pen is down (marking the paper) and the paper is moving.

(d) *Inject the sample onto either column A or column B following instructions.* Hold the syringe level and push the needle completely into the injector. Once the needle is no longer seen, quickly push the plunger and then pull the syringe out of the injection port.

Precautionary notes on Injection:

a) The injectors are very hot, so be careful not to touch the silver disk.

b) The needle will pass through a rubber septum, so one should feel some resistance. For some GC's, the column does not align properly in the injector, so the needle hits the front of the metal column. If one feels that he/she is pushing against metal, pull the needle out of the injector and try again, perhaps at a slightly different angle. The needle should completely disappear into the injector for proper injection of the sample onto the GC column.

c) Inject quickly for best results. Do not hesitate to inject once the needle is properly positioned in the injection port.

d) Remove the syringe immediately after injection. (Carrying out notes c and d helps to insure that the entire sample enters the GC column at approximately the same time.)

(e) *Mark the injection time on the chart recorder.* This can be done by adjusting the zero just after the sample is injected. It is often convenient for one person to inject the sample while a lab partner marks the injection time at the chart recorder.

(f) *Clean your syringe immediately after injection.* Syringe needles often clog quickly and must be replaced if they are not cleaned after each use.

(g) *Record the settings of your chart recorder during a run.* One needs to know the chart speed and the full-scale setting.

(h) *Record the settings of your GC during a run.* A knob on the bottom center of the GC can be turned to read column (or oven) temperature, detector temperature, and injector port temperature in $^{\circ}\text{C}$. The bridge current is displayed in milliamperes (mA). Note that there are two scales on the display. Be careful to read the appropriate scale!

2.3.2.1.2 Analysis of the Gas Chromatograph

Report the retention time of each peak (in minutes), the identity of each component in the mixture, and the percent composition of the mixture. To determine the percent composition, one will first need to find the area under each curve (see Section 3.1.2.3.1).

Mark retention time, height, half ($\frac{1}{2}$)-height, and width at $\frac{1}{2}$ height on the GC trace. Show calculations either in the final report or directly on the chromatograph.

It may be assumed that each component of the mixture causes the same response in the detector. Therefore, the areas under the curves can be used to calculate percent composition of the mixture of phthalate esters (this is a reasonable assumption when the components of the mixture are very similar in structure, as are diethyl phthalate and dibutyl phthalate).

Samples will have at least one solvent present, so one will see another peak in the GC traces that typically appears very soon (usually within a minute) after injection. It is normal for the solvent peak to go off scale.

2.3.2.2 Thin-Layer Chromatography (TLC)

Thin-Layer Chromatography (TLC) is a simple and inexpensive technique that is often used to judge the purity of a synthesized compound or to indicate the extent of progress of a chemical reaction. In this technique, a small quantity of a solution of the mixture to be analyzed is deposited as a small spot on a TLC plate, which consists of a thin layer of silica gel (SiO_2) or alumina (Al_2O_3) coated on a glass or plastic sheet. The plate constitutes the stationary phase. The sheet is then placed in a chamber containing a small amount of solvent, which is the mobile phase. The solvent gradually moves up the plate via capillary action, and it carries the deposited substances along with it at different rates. As the solvent rises by capillary action up through the adsorbent, differential partitioning occurs between the components of the mixture dissolved in the solvent, the stationary adsorbent phase. The more strongly a given component of a mixture is adsorbed onto the stationary phase, the less time it will spend in the mobile phase and the more slowly it will migrate up the plate. The desired result is that each component of the deposited mixture is moved a different distance up the plate by the solvent. The components then appear as a series of spots at different locations up the plate. Substances can be identified from their so-called R_f values. *The R_f value for a substance is the ratio of the distance that the substance travels to the distance that the solvent travels up the plate.* For example, an R_f value of 0.5 means that the spot corresponding to the substance travels exactly half as far as the solvent travels along the plate.

The Process of TLC. The steps involved in performing a TLC analysis include: preparing a spotting capillary; marking the TLC plate; spotting the TLC plate; developing the TLC plate; drying the plate; visualizing the substance spots, and measuring the R_f values.

Measurement of R_f . The distance between the 2 horizontal pencil lines is the distance of solvent advance. The distance from a bottom pencil line to the center of a substance spot is the distance of advance of the substance. The *ratio* of substance advance distance to solvent advance distance is the R_f value for the substance.

The following are some *common uses* of Thin-Layer Chromatography:

- ✓ To determine the number of components in a mixture.
- ✓ To determine the identity of two substances.

- ✓ To monitor the progress of a reaction.
- ✓ To determine the effectiveness of a purification.
- ✓ To determine the appropriate conditions for a column chromatographic separation.
- ✓ To monitor column chromatography.

The procedures can be used for qualitative confirmation of metabolite identity and for quantification of the compounds in samples directly spotted or prepared by existing procedures.

§2.4 Review of Literatures related to Test Microorganisms

As a result of the similarities of the basic metabolic processes of all living things and microorganisms are rather simple living systems which reproduce quickly and can be treated and studied easily, research on the metabolism of microorganisms is not only significant in itself, but is also an additional tool for the study of other living things. Thus, a study of the biodegradation of the PAEs by microbial systems is important to our understanding of the properties of these compounds. However, at present, only little reports on the degradation of PAEs by microorganisms are available (Jianlong *et al.*, 1995; Brossa *et al.*, 2003; Chang *et al.*, 2004; Zeng *et al.*, 2004; Chang *et al.*, 2007). These studies are neither systematic nor complete. In some of these cases, both the cell numbers and the biomass methods were explored, which are the conventional microbiological techniques, on the other hand, these methods are tedious and less efficient, especially when investigating a large number of PAE-microbial samples. Since the organic substrates have biphasic effects on the growth of some cells from animals and plants, that is, with stimulation at lower concentrations, and inhibition at higher concentrations. Understandably, it could be deduced that there would be biphasic effects of these compounds on the growth of some microorganisms. Microcalorimetry is a useful tool that has been extensively used in medical and biological areas. It allows the study of biology at the molecular level as well as at the cellular level. Thus, by monitoring the heat effect with a sufficiently sensitive calorimeter, the metabolic processes of living cells can be studied by a direct method. The calorimetry can directly determine the biological activity of a living system and provide a continuous measurement of heat production, thereby give much information in both qualitative and quantitative ways (Tan *et al.*, 1999).

Previously, there were no reports on the effects or degradation of the PAEs on the growth of *E. coli*, *S. aureus* and *A. niger* by means of microcalorimetry. Since PAEs are one of the most important compounds among the organic pollutants, their biodegradabilities have examined on *E. coli*, *S. aureus* and *A. niger* using this method. During the past decades, microcalorimetry has been applied to the study on the metabolism in microorganisms to obtain useful information about the metabolic processes, and have drawn the conclusions that the thermal effects, like other parameters describing the growth of microorganisms, such as cell numbers and biomass, can be used as measures for the growth of microorganisms. Furthermore, it has been found that when microorganisms are activated under the influence of activators (Zeng *et al.*, 2004; Chang *et al.*, 2007; Liang *et al.*, 2008), they would metabolize and grow rapidly and give out more heat; on the contrary, when microorganisms are inhibited under the influence of inhibitors, they would metabolize and grow slowly and give out less heat (Jianlong *et al.*, 1995;

Brossa *et al.*, 2003; Chang *et al.*, 2004; Zeng *et al.*, 2004; Chang *et al.*, 2007), and there should be a correlation between energy mechanisms and the growth states of microbial systems. So it may be expected that there would be raised thermal effects for the growth of some microorganisms in the presence of the PAE substrates since they act as the activators for other living things.

2.4.1 *Escherichia coli* (*E. coli*)

E. coli is Gram-negative and non-sporulating. Cells are typically rod-shaped and are about 2 micrometres (μm) long and 0.5 μm in diameter, with a cell volume of 0.6 - 0.7 μm^3 . It can live on a wide variety of substrates. *E. coli* uses mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate, and carbon dioxide. Since many pathways in mixed-acid fermentation produce hydrogen gas, these pathways require the levels of hydrogen to be low, as is the case when *E. coli* lives together with hydrogen-consuming organisms such as methanogenesis or sulfate-reducing bacteria.

Optimal growth of *E. coli* occurs at 37 °C (98.6 °F) but some laboratory strains can multiply at temperatures of up to 49 °C. Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen and amino acids, and the reduction of substrates such as oxygen, nitrate, dimethyl sulfoxide and trimethylamine N-oxide.

Strains that possess flagella can swim and are motile. The flagella have a peritrichous arrangement. *E. coli* and related bacteria possess the ability to transfer deoxyribonucleic acid (DNA) via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population.

2.4.2 *Staphylococcus aureus* (*S. aureus*)

S. aureus is a facultative anaerobic, Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood sugar plates. The golden appearance is the etymological root of the bacteria's name; *aureus* means "golden" in Latin.

S. aureus is catalase positive (meaning that it can produce the enzyme "catalase") and able to convert hydrogen peroxide (H_2O_2) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. A small percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test: *S. aureus* is primarily coagulase-positive (meaning that it can produce "coagulase", a protein product, which is an enzyme) that causes clot formation while most other *Staphylococcus* species are coagulase-negative. However, while the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase (the most common organism in patients with nosocomial bacteremia is coagulase-negative staphylococcus). Incorrect identification of an isolate can impact implementation of effective treatment and/or

control measures.

2.4.3 *Aspergillus niger* (*A. niger*)

Fungi are eukaryotes that have cell walls composed of chitin with or without cellulose. They are ubiquitous and comprise an estimated 250,000 species, of which only about 150 have been shown to cause disease in humans. They are chemoheterotrophic saprophytes. Morphology remains an important tool for speciating fungi, especially filamentous fungi, although morphologic clues can also be useful in the identification of yeast species.

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. *A. niger* (black on the surface and white to yellow on the reverse) is the third most commonly isolated species among the 185 species of the genus *Aspergillus* (Collier *et al.*, 1998). *Aspergillus* spp. (e.g., *A. fumigatus*, *A. flavus*, and *A. parasiticus*) are well-known to play a role in three different clinical settings in man: (i) opportunistic infections; (ii) allergic states; and (iii) toxicoses but most important for industrial applications are some members of the group of black aspergilli (*A. niger* and *A. tubingensis*). *Aspergillus* is in general the most commonly isolated one in invasive infections. It is the second most commonly recovered fungus in opportunistic mycoses following *Candida*. Since *Aspergillus* spp. are found in nature, they are also common laboratory contaminants (Arikan *et al.*, 2001).

The black aspergilli have a number of characteristics which make them ideal organisms for industrial applications, such as good fermentation capabilities and high levels of protein secretion. In particular, the wide range of enzymes produced by *Aspergillus* for the degradation of plant cell wall polysaccharides is of major importance to the food and feed industry. Recently, several *Aspergillus* spp. have received increased interest as hosts for heterologous protein production (Davies, 1994). Kormelink *et al.* (1993) observed that the presence of the *A. niger* acetylxylan esterase enabled degradation of steamed birchwood xylan by three types of endoxylanase and a β -xylosidase, which could not degrade this substrate in the absence of the esterase, indicating the importance of this enzyme in xylan degradation.

Papagianni *et al.* (1999) demonstrated a close link between morphology (quantified by image analysis) and citric acid production by *A. niger*, and identified a morphology parameter that was used successfully to characterize the process performance.

It's against these foregone backgrounds (pathogenicity, allergy, biodegradability, etc) that *A. niger* was used in this research to determine if it can play a positive role in the biodegradation of PAEs.

CHAPTER THREE: METHODOLOGY

This chapter describes the research materials and methods employed for this research to realize the aim and objectives of the study. They include experimental procedures for the measurements by microcalorimetry, microbial cell surface properties (hydrophobicity and autoaggregation), biodegradation by GC, TLC, spectrophotometry, etc.

§3.1. Materials and Methods

3.1.1 Materials

3.1.1.1 Microorganisms

The PAE-degrading microorganisms employed in this study were wild types of *Escherichia coli* (a Gram-negative, facultative anaerobic and non-sporulating bacterium), *Staphylococcus aureus* (a Gram-positive, facultative anaerobic coccus), and *Aspergillus niger* (a filamentous ascomycete fungus) and were all provided by the National Key Laboratory of Agromicrobiology, Huazhong Agricultural University (Wuhan, People's Republic of China).

3.1.1.2 Media preparations

Peptone nutrient medium (commonly known as Luria Broth, LB, medium) was used to incubate the bacteria, *E. coli* and *S. aureus* and was prepared by adding 10 g peptone and 5 g sodium chloride (NaCl) to a 1.0 L measuring cylinder containing 5 g beef extract and was made to the mark with distilled water. The medium, at pH 7.2 (about the optimum pH 7 determined by Xu *et al.*, 2005), was then sterilized in high-pressure steam in an autoclave at 121 °C for 30 min.

Potato sucrose medium (PSM), used to incubate the fungus, *A. niger*, was prepared by adding 20 mg D-glucose to a filtrate, obtained by boiling 200 mg peeled potato slices in 1.0 L distilled water for 30 min. The volume of the medium was adjusted to 1.0 L with distilled water, with no pH adjustment; and was sterilized in high-pressure steam in an autoclave at 121 °C for 30 min.

3.1.1.3 Preparation of microbial cell suspensions

For inoculum preparations, a loop full of each of the microorganisms was sub-cultured by inoculating them into 10 mL each of their respective sterile medium and incubated on a horizontal orbital shaker (FM FUMA, PR China) at 37 °C with orbital shaking 200 rev min⁻¹ (rpm) for 12–18 h, prior each measurement. The culture was then used as a source of inoculum for the experiments carried out in this work.

3.1.1.4 Reagents and sample preparations

Liquid hydrophobic hydrocarbon, n-octane (obtained from Sinopharm Chemical Reagent Company Limited, PR China) and two PAEs [diethyl phthalate (DEP) and dibutyl phthalate (DBP); obtained from Tianjin Standard Science and Technology Company Limited, PR China] were used in this work. A 5.0 mL stock solution of 5000 µg mL⁻¹ of each analytical grade 99.0% DEP and 99.5% DBP was prepared by thoroughly mixing 0.0223 mL DEP with 4.978 mL ethanol (95%±5); and 0.0239 mL DBP with 4.976 mL ethanol, respectively and stored in the refrigerator.

3.1.2 Methods

3.1.2.1. Microcalorimetric method

Enthalpy changes of microbial growth processes can be computed through microcalorimetry, quantifying continuously the exchange of heat between the growth system and the environment. In this study, a TAM III (the third generation thermal activity monitor) multi-channeled microcalorimeter (Thermometric AB, Järfälla, Sweden) in the isothermal mode was employed to measure continuously the heat production rate from microbial respiration (which is measured as microbial activity) or thermal activities of the wide variety of processes taking place in the test samples (mixtures of culture medium and various concentrations of the compounds, 0 – 300 µg mL⁻¹) contained in a series of cleaned and sterilized 4.0 mL stainless steel ampoules placed in the instrument in terms of heat, heat flow and heat capacity (Wadsö, 1997; Yao *et al.*, 2008) at 28 °C. Each of the hermetically

closed (by Teflon sealing discs) sample ampoules contained 2.0 mL of nutrient medium, various doses of PAE, a loop of cell suspension and 2.0 mL of air/headspace. The aim of the ampoule closure was to ensure the control of evaporation yet allowing oxygen and carbon dioxide transfer (Crittter and Airoidi, 2001). The cell concentration of inoculums for bacteria and fungus was controlled equally for the purpose of comparison. The culture medium (i.e., 2.0 mL nutrient medium plus microorganisms) in the reference ampoule served as blank (or control) in all the determinations and once the system was cleaned and sterilized and the baseline has been stabilized, all PAE cell suspensions were loaded into the microcalorimeter.

The values of the total heat generated or total thermal effect (Q_T), peak-heat output power (P_{Max}), and peak-time [t_{Max} , related to the maximum position on the P-T curves shown in Figs. 1(a)-(c) and 2 (a)-(c)] of the growth process for each experiment (from the start of the growth-process until data collection was ended) was computed by integrating the area limited by the Power(P)-Time(T) curves (Prado and Airoidi, 2000; Crittter *et al.*, 2001; Crittter *et al.*, 2002). Similarly, the microbial growth rate constant (k) and the generation time (t_G) were determined from the P-T curves. The quantification procedures for these parameters were well established in Hashimoto and Takahashi (1982). The thermogenic (P-T) curves generated from all the experiments illustrated typical patterns of all the phases of the exponential microbial growth-process reactions in environmental matrices (Kimura and Takahashi, 1985; Nuñez *et al.*, 1994): *lag*, *exponential/log growth* (exponential increase of the heat evolution rate), *stationary* and *death phases* (decline of the P-T curve due to microbial death) and hence obey the thermokinetic relation (Equation 1) at the log phase of growth, $S_t = S_o \exp(-kt)$

(1)

where S_o and S_t represent concentrations of DEP or DBP at times 0 and t , respectively. The biodegradation kinetics of organic compounds has been described by so many models and the first-order kinetics has been often utilized to describe their biodegradation process at low concentrations (Zeng *et al.*, 2004). The *degradation rate* of the PAE by the microbes, expressed as *relative rate of degradation* (δ), could be determined using S_t and S_o [Equation (1)] via Equation (2) as the DEP or DBP dose utilized divided by the original DEP or DBP dose, multiplied by 100.

$$\% \text{ PAE degradation } (\delta) = [1 - (S_t/S_o)] \times 100\% = (\Delta S/S_o) \times 100\%, \quad (2)$$

The *half inhibitory concentrations*, IC_{50} , of these compounds (an inhibitory concentration capable of causing a 50% decrease of microbial growth rate) were obtained from the microcalorimetric method by interpolation regression analysis using OriginPro 8.0 (see Section 3.2) at 50% of the growth inhibitory ratios (I), calculated using Equation (3).

$$I = [(k_0 - k_C)/k_0] \times 100\%, \quad (3)$$

where k_0 and k_C are the growth rate constants of the blank (or control, 0.0 $\mu\text{g mL}^{-1}$) and of the PAE-containing samples at a concentration of C , respectively.

3.1.2.2 Shake flask experiments

In order to investigate the extent to which a range of concentrations of DEP or DBP (0.0, 50, 100, 200, and 300 $\mu\text{g mL}^{-1}$) could be used as a carbon and energy source for the microbes under aerobic growth conditions, shake flask experiments were performed. Each culture was grown individually in 150 mL of respective culture medium (composed of 3.0 mL free cell inoculum plus 147 mL sterile nutrient medium) in 250 mL Erlenmeyer flask on the same shaker used for inoculum preparation with PAE (*test culture*) or without PAE (*control culture*). To ameliorate or prevent background interferences or data misinterpretations from concomitant color development or precipitation formation in spectrophotometric analyses, each assay also had the following blanks: for *control cultures*, blanks composed of 3 mL sterile distilled water plus 147 mL nutrient medium (i.e., *blank/control 1*); and for *test cultures*, blanks composed of respective concentrations of test compound plus 150 mL nutrient medium (i.e., *blank/control 2*). These blanks were also employed to measure the true given concentration by the GC (see Section 3.1.2.3.1) for the confirmation of the concentration in the culture medium.

Flasks were taken in turn at predetermined times and assayed for cell autoaggregation ability measurements (see Section 3.1.2.2.1) at the end of the 24-h incubation of the flasks in dark (*to prevent reaction of the PAEs with light; photolysis*) at 28 °C and 120 rpm. The remaining culture medium was centrifuged, using TGL-16M (PR China) centrifuge, at 5000 rpm for 20 min to obtain cell pellet, which was used to evaluate the effect of DEP or DBP on microbial CSH (see Section 3.1.2.2.2).

Furthermore, portions of the contents of these flasks were simultaneously analyzed for their *optical densities* (*ODs*) (see Section 3.1.2.2.3) and *biomass* (see Section 3.1.2.2.4) at time, $t = 0$ h, and other predetermined times.

3.1.2.2.1 Cells autoaggregation assay

Portions of the contents of flasks (from the experimental set-up in Section 3.1.2.2) containing the control, 50 $\mu\text{g mL}^{-1}$ PAE and the blanks were taken in turn at predetermined times and assayed for cell autoaggregation ability at the end of the 24-h incubation period by vortexing for 15 s and allowed to stand at room temperature for 120 min. The absorbance change at 660 nm of 3.5 mL inoculated culture medium at 10 min intervals were monitored by a Spectrum Lab 725s Spectrophotometer (Lingguang Ltd., Shanghai, PR China). All measurements were done in triplicates and their arithmetic means were used for calculations. Autoaggregation ability was determined as percent autoaggregation (% A_{Ag}) using the following equation:

$$\% A_{Ag} = [1 - (A/A_o)] \times 100 \quad (4)$$

where A_o and A are the absorbances of the cultured media at 0 and 120 min intervals, respectively. Microbes with $\%A_{Ag} \geq 70\%$, 50–70% and $< 50\%$ can arbitrarily be designated as highly, moderately and low autoaggregating, respectively.

3.1.2.2.2 Microbial cell surface hydrophobicity (CSH) assay

Hydrophobicity of the microbial cell suspensions prepared as above was determined, using MATHS (microbial adhesion to hydrocarbons) assay, as a measure of their adherence to the hydrophobic hydrocarbon (n-octane) following the procedure described previously by Rosenberg *et al.* (1983). After 24-h incubation, microbial cells were concentrated and harvested during the exponential growth phase by centrifugation (5000 x rpm for 20 min, TGL-16M, PR China); washed twice with phosphate buffered saline (PBS; 7.6 g NaCl, 1.9 g Na₂HPO₄·7H₂O and 0.7 g NaH₂PO₄·2H₂O per liter, pH 7.2), resuspended in the same buffer and the absorbance measured at 660 nm (A_1). 5.0 mL of microbial suspension and 1.0 mL n-octane (i.e., the assay mixture) were mixed for 120 s by vortexing to ensure thorough mixing and then incubated for 1 h without shaking to insure that the two solutions separated into biphasic state. The absorbance of the lower hydrophilic (aqueous) layer was measured again (A_2) by recording the changes in absorbance of microbial suspensions due to microbial adhesion to n-octane at 660 nm using the spectrophotometer. All measurements were done in triplicates and their arithmetic means were used for calculations. Microbial cell surface hydrophobicity was expressed as percentage adherence (% *Adh*) and calculated using the following equation:

$$\% Adh = [1 - (A_2/A_1)] \times 100 \quad (5a)$$

Microbes with % *Adh* $\geq$ 70%, 50–70% and < 50% can arbitrarily be classified as highly, moderately and low hydrophobic, respectively.

3.1.2.2.3 Optical Density (OD_{660})

Cell growth was monitored by measuring the optical density of culture samples (0.0, 50, 100, 200, 300 $\mu\text{g mL}^{-1}$) prepared in Section 3.1.2.2 spectrophotometrically at the wavelength of 660 nm (OD_{660}). All measurements were done in triplicates and their arithmetic means were used to plot Fig. 4.11. Growth inhibition analysis was done using the OD_{660} values and toxic effects were calculated by quantifying the extent of growth inhibition in the test cultures (OD_T , the mean OD_{660} values for 50–300 $\mu\text{g mL}^{-1}$ DEP or DBP; see Appendices 11 and 12 a – f and Table 4.5) in comparison with the control cultures (OD_C , the mean OD_{660} values for 0 $\mu\text{g mL}^{-1}$ DEP or DBP; see Appendices 11 and 12 a – f and Table 4.5) after the incubation time. As a consequence, percent inhibition ratio (%*I*) was calculated using Equation (5b) and IC_{50} was determined by interpolation regression analysis using OriginPro 8.0.

$$\%I = (OD_C - OD_T)/OD_C \times 100 \quad (5b)$$

each experiment consisted of one control and up to three test cultures with inhibition scores between >0% - <100%.

3.1.2.2.4 Biomass (cell dry weight) measurements

In each measurement, 10 mL samples of the 200 $\mu\text{g mL}^{-1}$ growth culture (from Section 3.1.2.2) were centrifuged

at 5000 x rpm for 20 min in a pre-weighed tube. For each test solution, the top clear liquid was filtered and the cell residue or the biomass was then dried for 12 h at 100 °C and weighed to determine the growth culture dry weight (dw). All measurements were done in triplicates and their arithmetic means were used for plotting Fig. 4.10.

3.1.2.3 Chromatographic methods

3.1.2.3.1 Gas Chromatography of the biodegradation of PAEs

125 mL Erlenmeyer flasks were soaked overnight in a bath of concentrated sulphuric acid containing $\text{K}_2\text{Cr}_2\text{O}_7$, and then rinsed with distilled-deionized water. Flasks containing 49 mL 0.05 mol L^{-1} sterile potassium phosphate buffer (pH 7.2) were autoclaved and $5000 \mu\text{g mL}^{-1}$ of each of DEP and DBP solutions (0.0, 50, 100, 200, 300 $\mu\text{g mL}^{-1}$) were simultaneously added and then inoculated with 1 mL cells (of either *E. coli*, *S. aureus* or *A. niger*) suspended in sterile 0.05 mol L^{-1} potassium phosphate buffer (pH 7.5) and the flasks were incubated in darkness (*to prevent reaction of the PAEs with light; photolysis*) at 28 °C on a rotary shaker for 6 h at 120 rpm. The cell density (biomass) of inoculum for the microbes was controlled to be equal for the purpose of comparison. Periodically, 10 mL portions of n-hexane were added directly to individual flasks which were vigorously shaken for 10 min and then left standing to allow the solvent and aqueous phases to separate. The solvent phase was then removed. This supernatant extraction step was repeated three times to extract the two PAEs. Aliquots of the solvent phases were mixed, dried with Na_2SO_4 , and volatilized. Two milliliters (2.0 mL) residues were dissolved in 2.0 mL portions of n-hexane, and a 2- μL portion of this solution was injected into a gas chromatograph (Hewlett-Packard Model 5980; J.W., USA). The column (thickness, $0.25 \mu\text{m}$; inner diameter, 0.25 mm; length, 15 m) was maintained at 250 °C, and the flow rate of the N_2 carrier gas was 60 mL min^{-1} . The injection and detector temperatures were 250 and 300 °C, respectively. For each of the two PAEs (DEP and DBP), the detection limit was $100 \mu\text{g L}^{-1}$. This procedure was performed on samples in which uninoculated inorganic salts solution amended with various concentrations of phthalate were used in place of the cultures to obtain standard peak areas. In order to also eliminate the possibility of PAEs decomposition by chemical reactions such as hydrolysis and oxidation, their degradations were examined using sterile controls (for both PAEs) of uninoculated media incubated/autoclaved with the other flasks. Data collection and processing were carried out using MSD Productivity Chemstation software (Hewlett Packard) containing the Wiley chemical library.

To determine the percent composition, firstly, the area under each curve was calculated using Equation (6a)

$$\text{Area} = (\text{height}) \times (\text{width at } \frac{1}{2} \text{ height}) \quad (6a)$$

It was assumed that each component of the mixture caused the same response in the detector. Therefore, the areas under the curves were used to calculate percent composition (Equations 6b and 6c) of the mixture of phthalate esters (this is a reasonable assumption since the components of the mixture are very similar in structure). The analyses were carried out in triplicate and the mean amount of each compound was expressed as a relative percentage of the total area of the chromatograms.

$$\% \text{ Component 1} = [(\text{area under peak 1}) / (\text{total area})] \times 100\% \quad (6b)$$

$$\rightarrow \text{Relative PAE residue dose} = \text{dose of residue} / \text{initial dose of PAE} = C/C_o \quad (6c)$$

3.1.2.3.2 Thin-Layer Chromatography (TLC) Assay (Analyses of metabolites)

The biodegradations of these compounds involve multiple degradation steps, and metabolic intermediates (metabolites) are often accumulated in the media (Guerin and Jones, 1988; Kiyohara and Nagao, 1978). Therefore, it became necessary to analyze these metabolites in order to completely identify the intermediates/end products of these degradations.

The intermediates of DEP or DBP degradation were analyzed by *double extraction* of the residual compounds and its metabolites from the culture solution with 25 ml dichloromethane and 15 ml petroleum ether at 40-60 °C after acidification of the medium with 2N HCl. Samples were centrifuged and supernatants used for analysis of metabolites and substrates. The combined extracts were dried with anhydrous Na₂SO₄ and used for quantitative thin-layer chromatography (TLC) on silica gel plates using a 9:1 ratio of benzene/acetic acid as developing solvent. Reference substances and metabolites were detected under ultraviolet (UV) light 275 nm.

$$R_f = \text{substance advance distance} / \text{solvent distance} \quad (7)$$

When the solvent front moved to within about 1 cm of the top end of the adsorbent (after 15 to 45min), the plate was removed from the developing chamber, the position of the solvent front marked, and the solvent allowed to evaporate. Because the components (metabolites) were colorless, they were visualized by shining UV light 275 nm on the plate. This experiment was done in triplicate and the mean R_f values for the references (i.e. standard DEP and DBP) and the metabolites were obtained for the purpose of identifying the degradation metabolites by comparing the two sets (reference and metabolite) of R_f values.

§3.2 Statistical analyses

Experimental data were obtained through three parallel experiments. Means and standard deviations of triplicates were determined using Originlab Scientific Graphing and Analysis software (OriginPro8.0). This tool was also used to evaluate the differences between cultures, and the biodegradability, effect of DEP or DBP by statistically determining the significance at $P < 0.0001$ level of difference between treatments and the correlation coefficient ($r > 0.99$). Correlations were tested using the Pearson's correlation coefficient.

CHAPTER FOUR: RESULTS AND DISCUSSIONS

The results of the chemical analyses for PAEs in liquid culture medium samples are presented and discussed here. Except otherwise stated, concentrations of PAEs in prepared samples were expressed in microgram per milliliter ($\mu\text{g mL}^{-1}$).

The results from microcalorimetric measurements were presented and discussed in Section 4.1, those from the “Shake Flask” experiments in Section 4.2 and those from the chromatographic assays were presented in Section 4.3. The relationships between/among the data from these different analytical methods were discussed in Section 4.4.

The discussion is an exercise presented in logic and disciplined fashion. This section stated the main findings of the study with respect to the methods, and addressed possible inadequacies; compared the results with other published findings; and discussed the implications of the results. This part of the work also answers the questions: “What does this mean for practice of policy?” and “Are there any needs for further studies?”

§ 4.1 Microcalorimetry

4.1.1 The growth thermogenic curves at various doses of DBP

The P - T curves for the growth of *E. coli*, *S. aureus* and *A. niger* in the presence of different doses of DEP and DBP (individually used as the sole carbon and energy source) at 28 °C are respectively shown in Fig. 4.1 and Fig. 4.2. All of them depicted more or less, a typical growth curve for microbes and can be divided into four phases: lag, log/exponential, stationary, and decline/death phases. If the heat output power P_0 and P_t are the heat output powers at time = 0 and time = t , respectively, then:

$$P_t = P_0 \exp(kt) \text{ or } \ln P_t = \ln P_0 + kt \quad (8)$$

All the power–time curves displayed a typical process of microbial metabolic activity (Yao *et al.*, 2008). The growth rate constant (k) can be obtained from the rise part of the first peak by plotting a linear curve of $\ln P_t$ against t and is summarized in

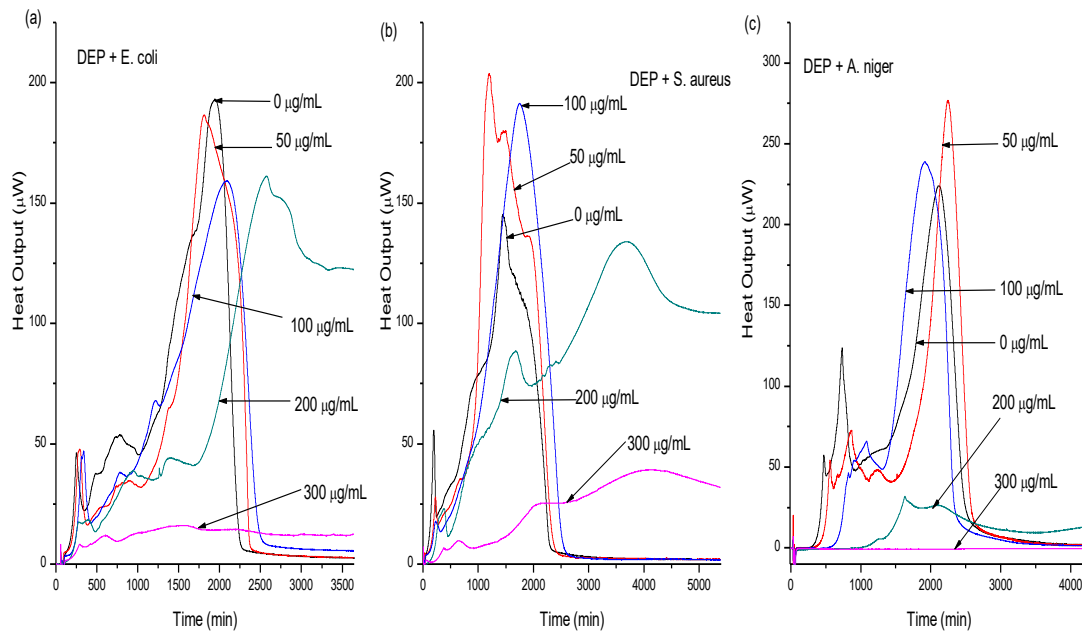


Fig. 4.1. The growth thermogenic (power-time, P-T) curves of (a) *E. coli* in Luria Broth (LB) medium, (b) *S. aureus* in LB medium and (c) *A. niger* in potato sucrose medium (PSM) affected by varied doses of DEP at 28 °C.

Tables 4.1 and 4.2. Fig. 4.1 and Fig. 4.2 show that these PAEs had an obvious inhibitory action on the growth of these microbes. The IC_{50} value for the effect of each compound on the microbes was determined at 50% of the inhibitory ratio (I) from Equation (3) and by extrapolating the concentration (C) versus I plot (suppose to be linear relationship). Some similarities and differences can be observed from a qualitative point of view. The power–time curves in the presence of the PAEs can still be divided into four phases, in which the lag phase, log phase and decline phase are very similar to, but the stationary phases are significantly different from those of the microbes without PAEs. During the lag phase and the log phase for the microbes, PAEs have the capacity to inhibit its growth to a different extent and the inhibitory effects increase with the concentrations of DEP or DBP. The time of the lag phase suggested that the retarding times of these microbes are longer with the increasing

concentrations of PAEs. During the stationary phase of the organisms in the presence PAEs, the heat given out is greater than that of microbes without PAEs.

4.1.2 Inhibition and Tolerance

The k , I and IC_{50} values are presented in Tables 4.1 and 4.2 with correlation coefficients (r) greater than 0.99. Inhibitory ratio (I) is important index to evaluate toxicological effect of PAEs and was each calculated for each microbe in relation to these compounds (see values in Tables 4.1 and 4.2). The k values, decreasing with increase in the DEP or DBP doses [see Fig. 4.3 (a) - (f)], show that these compounds had potential antimicrobial activity even though they were almost completely used-up in these experiments (see δ in Tables 4.1 and 4.2). The IC_{50} values (*E. coli*, 197.37 $\mu\text{g mL}^{-1}$; *S. aureus*, 154.43 $\mu\text{g mL}^{-1}$; and *A. niger*, 127.84 $\mu\text{g mL}^{-1}$) in Table 4.1 indicate that *A. niger* and *E. coli* are the most sensitive and most tolerant microbes to DEP, respectively; and those (*E. coli*, 154.43 $\mu\text{g mL}^{-1}$; *S. aureus*, 137.95 $\mu\text{g mL}^{-1}$; and *A. niger*, 155.68 $\mu\text{g mL}^{-1}$) in Table 4.2 indicate that *A. niger* and *S. aureus* are the most tolerant and most sensitive microbes to DBP, respectively. From analysis of the power–time curves for the microbes in Fig. 4.1 and Fig. 4.2, it can be seen that the heat power during the log phases of the curves were increased exponentially. So the growth rate constants (k) of microbes, obeying the equation: $P_t = P_0 \exp(kt)$ or $\ln P_t = \ln P_0 + kt$ can be calculated. According to the equation, the values of k and the

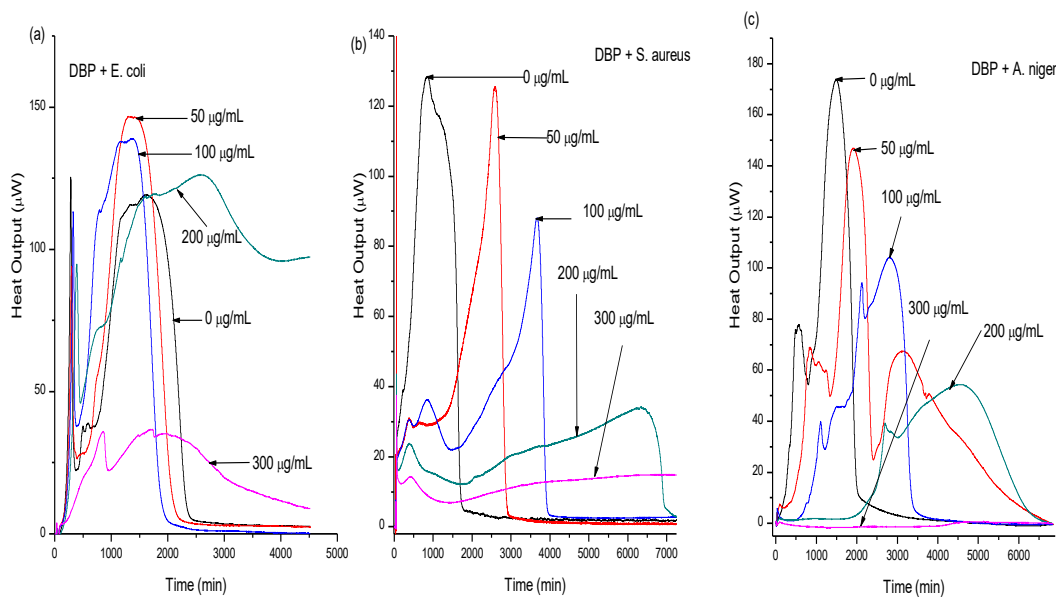


Fig. 4.2. The growth thermogenic (power-time, P-T) curves of (a) *E. coli* in Luria Broth (LB) medium, (b) *S. aureus* in LB medium and (c) *A. niger* in potato sucrose medium (PSM) affected by varied doses of DBP at 28 °C.

correlation coefficients, R , were calculated using OriginLab Pro 8.0 and are listed in Table 4.3. From Table 4.1, Table 4.2 and Table 4.3, it was observed that the rate constants decrease with the increasing concentrations of PAEs. Figure 4.3 shows the relation of the rate constants and concentrations of PAE substrates. When the concentrations of PAEs reached 300 $\mu\text{g mL}^{-1}$, the PAEs exerted complete inhibitory effects on the growth of the microbes, in this case no thermal effects can be observed in the power–time curve during the experimental time.

4.1.3 Degradation Efficiencies

The DEP degradation efficiencies (δ) recorded in Tables 4.1 and 4.2 for *E. coli*, *S. aureus*, and *A. niger* are 51.9 - 93.5, 91.9 - 99.8 and 59.8 - 99.9%, respectively; deducing that *S. aureus* (mean $\delta = 96.18\%$) is the most efficient DEP degrader and *E. coli* (mean $\delta = 83.83\%$) the least and those for DBP for *E. coli*, *S. aureus*, and *A. niger* are 63.8 - 99.1, 13.7 - 98.5 and 92.3 - 100.0%, respectively; inferring that *A. niger* (mean $\delta = 97.27\%$) is the most efficient DBP degrader and *S. aureus* (mean $\delta = 75.95\%$) the least.

With increasing DEP or DBP doses the time for the appearance of the peaks on each curve (i.e., t_{max} values; see Tables 4.1 and 4.2; and Fig. 4.1 and 4.2) increased with a corresponding decrease in their heights (i.e., P_{max} and Q_T values; see Tables 4.1 and 4.2; and Fig. 4.1 and 4.2) with little or diminishing peaks on curves for higher doses (200 and 300 $\mu\text{g mL}^{-1}$) of DEP and DBP. These trends suggest that DEP and DBP display inhibitory effect on the microbes with the increase in their dosages, which consequence could likely be attributed to the increased maintenance requirements due to irreparable harm to the cellular envelope, as was observed by Tiehm (1994) and Chen *et al.* (2000). The growth curves of these microbes have two characteristic peaks, suggesting that these microbes might have adopted two ways of metabolism. The presence or absence of oxygen (O_2) is very significant for microbial growth, hence influencing the ways of microbial metabolism. In this experiment, the volume of O_2 /the headspace in the ampoule is approximately 2 mL and this is only a

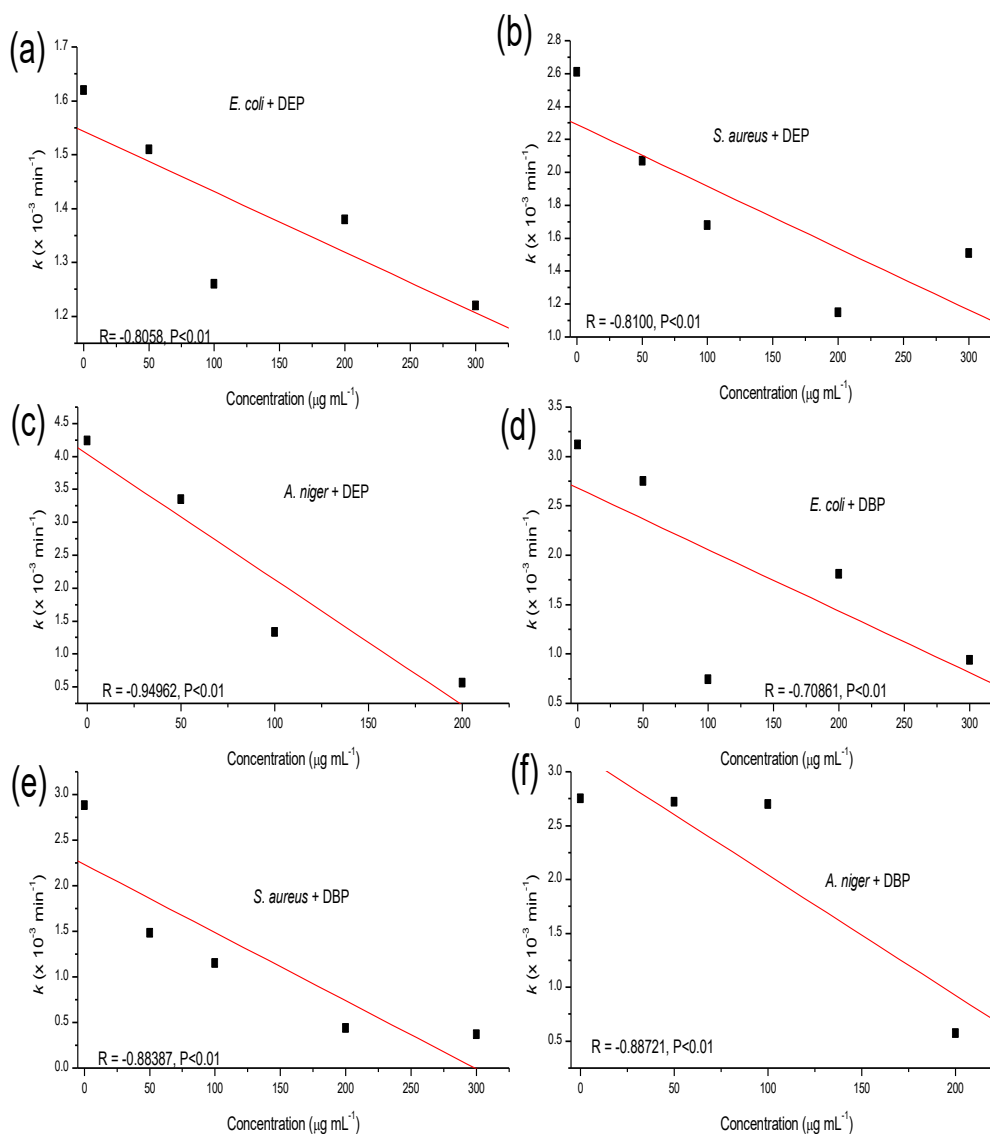


Fig. 4.3. Relationship between the microbial growth rate constants (k) of the three microbes and the various concentrations of DEP [(a), (b), (c)]; and DBP [(d), (e), (f)].

Table 4.1. Enumerative microcalorimetric experimental results* of the effects of increasing doses of DEP on the growth of the three microbial strains at 28 °C.

D	m	P	t	Q	k		t	M	DE	I	I
o	o	m	m	T	(		G	ole	P	(	C
s	l	ax	a	(	1	R	(	sD	De	%	50
e	e	(	x	J	0		m	EP	gra	)	(
(	s	μ	(	)	-		i	(Δ	dati		μ

μ g m L ⁻¹) (C)	D E P (S o) (1 0 - 4 m o l) L ⁻¹	W)	m i n)		³ m i n - l)		n)	S) (1 0 ⁻⁴ m ol) L ⁻¹	on (δ) (%)		g m L ⁻¹)
<i>Escherichia coil</i>											
0 5 0 1 0 0 2 0 0 3 0 0	0 . 0 2 . 2 5 4 . 5 0 9 .	1 9 3 . 2 1 8 6 . 7 3 1 5 9 .	1 9 4 4 1 8 0 2 0 9 0 2 5 7 4	1 0 . 0 1 . 0 7 1 0 . 2 9 1	1 . 6 2 1 . 5 1 1 6 1 . 3 8	0 . 9 9 6 5 0 . 9 2 8 0 . 9 9	4 2 8 4 5 9 5 5 0 2 5 6 8	0 . 0 0 2 . 1 0 4 . 1 8 8 . 7 4	0 . 0 9 3 . 5 9 2 7 1 5	0 . 0 6 . 7 9 2 . 4 . .	1 9 7 . 3 7

	1 3 . 5 0	3 7 1 6 1 . 2 9 1 2 . 0 0	6 0 0	4 . 4 1 0 . 2 0	1 . 2 2	8 3 0 . 9 9 8 3 0 . 9 9 6 0		7 . 0 1	1 . 9	8 1 2 4 . 6 9	
<i>Staphylococcus aureus</i>											
0 5 0 1 0 0 2 0 0 3 0 0	0 . 0 0 2 . 2 5 4 . 5 0 9 . 0 0 1 3 . 5 0	1 4 5 . 2 0 3 . 8 1 1 3 9 1 . 4 8 1 1 3 6	1 4 3 9 1 1 7 5 1 3 6 8 4 1 0 6	9 . 7 6 1 2 . 9 7 1 2 . 7 2 7 . 5 5 1 5 7 .	2 . 6 1 2 . 0 7 1 . 6 8 1 . 5 1 5 1	0 . 9 9 9 0 9 9 0 . 9 9 1 1 0 . 9		0 . 0 0 2 . 0 6 4 . 2 6 8 . 8 7 1 3 . 4 7	0 . 0 0 9 1 . 6 9 8 . 6 9 . 8 9 . 8	0 . 0 0 2 0 . 6 9 3 5 . 5 6 4 4 2	1 5 4 . 4 3

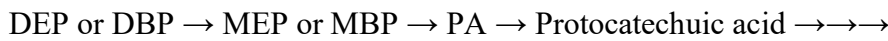
		. 1 0 3 9 . 2 7		5 2		9 0 5 0 . 9 9 8 7				. 1 5	
--	--	--------------------------------------	--	--------	--	---	--	--	--	-------------	--

<i>Aspergillus niger</i>											
--------------------------	--	--	--	--	--	--	--	--	--	--	--

		2 2 4 . 2 5 2 7 7 . 0 7 1 2 3 9 9 . 0 0 8 3 2 . 5 0 6 -		1 3 . 0 3 1 2 . 0 5 1 1 1 4 1 6 2 7 3 3 -		0 . 9 9 5 9 0 2 4 3 5 1 3 0 . 5 6 3 -		0 . 9 9 1 6 0 2 9 8 6 2 0 8 0 0 - 9 9 7 8 -		0 . 0 2 0 . 9 9 9 2 4 5 9 2 8 - 0 0 1 0 0	
--	--	--	--	---	--	---	--	--	--	---	--

* See Appendices for results written in the form “**mean±standard deviation**”

limited amount of O₂ to be consumed by the microbes in the system. The cells adopted one way of metabolism (aerobic) at the start of the experiment and when the O₂ was used up, the microbes may have adjusted themselves and adopted another metabolic way (anaerobic) which may explain the presence of two peaks. It is interesting to note that the heights of the second peaks increased greater than the first at low and medium DEP or DBP doses and little or diminishing peaks on curves for higher DEP or DBP doses (200 and 300 µg mL⁻¹). Therefore, it may be concluded that the first way of metabolism has been influenced greater than the second way and that in the second way of metabolism, these facultative microbes must have consumed the DEP or DBP and/or subsequently its related biodegradation intermediates (monoethyl phthalate/monobutyl phthalate, MEP/MBP; phthalic acid, PA; and protocatechuic acid) that may have accumulated during the primary degradation of DEP or DBP, as sources of carbon and energy needed for their continued growth for a definite time. Biodegradation of PAEs (DEP or DBP) (Kurane *et al.*, 1978; Wang *et al.*, 1995), respectively, follows the proposed degradation pathway:



involving systematic cleavage of the ester bond to produce the phthalate monoesters (MEP and MBP) and then PA which is further metabolized and goes through the tricarboxylic acid (TCA) cycle to produce carbon dioxide (CO₂) and water (H₂O) (Wang *et al.*, 1995).

4.1.4 DEP and DBP Biodegradation Kinetics

Several techniques have been used to describe the biodegradation kinetics of organic pollutants, and the first-order kinetics has been frequently used to express biodegradation progress at low substrate doses. The biodegradation of DEP and DBP by *E. coli*, *S. aureus* and *A. niger* can be assumed to fit to the first-order kinetic equation, which has the form:

$$\ln C = kt + A \quad (10)$$

where C = initial dose of DEP and DBP; k = first-order kinetic constant; t = time; A = constant.

The half-life of first-order biodegradation reaction of the DEP and DBP by *E. coli*, *S. aureus* and *A. niger* can be expressed as:

$$t_{1/2} = \ln 2/k = 0.6931/k \quad (11)$$

The half-lives and kinetic equations for the DEP and DBP biodegradations by the three microbes were listed in Table 4.3. The computed results indicated that the first-order model gives a better fit. The biodegradation kinetics of DEP and DBP by the microbes can be described by a first-order reaction model under the experimental initial doses. The rate constants (k) were independent of initial concentrations, and the mean first-order equations for *E. coli* + DEP, *S. aureus* + DEP, *A. niger* + DEP, *E. coli* + DBP, *S. aureus* + DBP, and *A. niger* + DBP could be expressed as: $\ln C = -0.00134t + A$, $\ln C = -0.00160t + A$, $\ln C = -0.00175t + A$, $\ln C = -0.00156t + A$, $\ln C = -0.00086t + A$, and $\ln C = -0.00200t + A$, respectively and their corresponding mean biodegradation half-lives were $t_{1/2}(\text{DEP}/E. coli) = 8.67$ h, $t_{1/2}(\text{DEP}/S. aureus) = 7.54$ h, $t_{1/2}(\text{DEP}/A. niger) = 10.92$ h, $t_{1/2}(\text{DBP}/E. coli) = 9.66$ h, $t_{1/2}(\text{DBP}/S. aureus)$

= 18.81 h, $t_{1/2}(\text{DBP}/A. niger)$ = 9.60 h, respectively. The first-order rate coefficient (k) decreased gradually, suggesting that the higher initial concentrations (200 and 300 $\mu\text{g mL}^{-1}$) of DEP and DBP hindered the biodegradation.

The biodegradability of the PAEs by these microbes appeared to be related to the length of the alkyl side chains. Relatively, the shorter-chain DEP was degraded at higher rate than the longer-chain DBP. It's observed that as the octanol/water

Table 4.2. Enumerative microcalorimetric experimental results* of the effects of increasing doses of DBP on the growth of the three microbial strains at 28 °C.

Dose ($\mu\text{g mL}^{-1}$) (C)	mole sDBP (S_o) ($10^{-4}\text{ mol L}^{-1}$)	P_{max} (μW)	t_{max} (min)	Q_{T} (J)	k (10^{-3} min^{-1})	R	t_{G} (min)	Mole sDBP (ΔS) ($10^{-4}\text{ mol L}^{-1}$)	DBP Degradation (δ) (%)	I (%)	I_{C50} ($\mu\text{g mL}^{-1}$)
<i>Escherichia coli</i>											

0	0	1				0					
5		2				.					
0		5				9					
1		.				9				0	
0	0	3		1		9				.	
0	.	2	2	0	3	4		0	0	0	
1	0	1	8	.	.	0		.	.	0	
0	0	4	2	0	1	.	2	0	0	1	
0	1	6	1	1	2	9	2	0	9	1	
0	.	.	3	.	2	9	2	1	7	.	
2	8	7	0	7	.	9	2	.	.	8	
0	0	9	4	7	7	7	5	7	2	6	
0	3	1	1	7	5	0	2	5	6	7	1
0	.	3	3	9	0	.	9	2	3	6	5
2	5	9	7	.	.	9	4	.	.	.	4
0	9	.	4	5	7	9	2	2	8	5	.
0	7	0	2	8	4	9	3	9	9	0	4
3	.	2	5	2	1	3	8	7	9	4	3
0	1	1	7	1	.	0	3	.	.	1	
0	9	2	5	.	8	.	7	1	1	.	
	1	6	1	7	1	9	4	2	8	9	
	0	.	7	9	0	9	1	8	0	9	
	.	3	2	5	.	8		.	.	7	
	7	5	8	.	9	7		6	3	0	
	8	3		4	4	0		6		.	
		6				.				0	
		.				9				3	
		8				9					
		0				7					
		0				9					
Staphylococcus aureus											
0	0	1	8	9	2	0	2	0	0	0	1
5	.	2	6	.	.	.	4	.	.	.	3
0	0	8	1	5	8	9	1	0	0	0	7
1	0	.	2	1	8	9	4	0	9	0	.
0	1	5	5	7	1	8	6	1	7	4	9

0	.	4	7	.	.	3	8	.	.	8	5
2	8	1	8	9	4	0	6	7	8	.	
0	0	2	3	8	8	.	0	6	9	6	
0	3	5	6	9	1	9	3	3	8	1	
3	.	.	5	.	.	9	1	.	.	6	
0	5	5	8	4	1	8	5	5	5	0	
0	9	9	6	2	5	6	7	4	9	.	
	7	8	3	7	0	0	9	6	3	0	
	.	8	2	.	.	.	1	.	.	7	
	1	.	0	5	4	9	8	7	8	8	
	9	1	3	3	4	9	7	4	1	4	
	1	9	9	0	0	9	8	1	3	.	
	0	3	7	.	.	2		.	.	7	
	.	4		7	3	0		4	7	6	
	7	.		6	7	.		7		8	
	8	3				9				7	
		0				9				.	
		1				8				1	
		4				4				9	
		.				0					
		3				.					
		6				9					
						9					
						8					
						6					

<i>Aspergillus niger</i>											
0	0	1	1	1	2	0	2	0	0	0	
5	.	7	4	0	.	.	5	1	9	1	
0	0	3	9	.	7	9	2	.	9	.	1
1	0	.	3	8	5	9	2	7	.	0	5
0	1	8	1	1	2	5	5	9	5	9	5
0	.	4	9	1	.	1	5	3	1	1	.
2	8	1	1	7	7	0	2	.	0	.	6
0	0	4	9	.	2	.	5	5	0	8	8
0	3	6	2	6	2	9	7	9	.	2	
3	.	.	8	4	.	9	1	6	0	7	

0	5	9	0	9	7	6	2	.	9	9	
0	9	9	5	.	0	4	0	6	2	.	
	7	1	4	8	0	0	8	4	.	1	
	.	0	5	5	.	.	-	-	3	3	
	1	4	0	8	5	9			-	1	
	9	.	0	.	7	9				0	
	1	3	-	9	-	8				0	
	0	1		7		2				.	
	.	5		-		0				0	
	7	4				.				0	
	8	.				9					
		5				9					
		0				8					
		-				3					
						-					

* see Appendices for results written in the form “**mean±standard deviation**”

partition coefficient (k_{ow}) increased and the water solubility decreased, the biodegradability of PAEs by these microbes declined. The order of the biodegradation of the two PAEs by these microbes wasDEP (*S. aureus*)> DBP (*A. niger*)> DEP (*A. niger*)> DEP (*E. coli*)> DBP (*E. coli*)> DBP (*S. aureus*). This trend was observed in Section 4.1.3 and also confirmed theobservation made by other reseachers, including

Table 4.3. Half-lives and kinetic equations of the DEP and DBP biodegradations by *E. coli*, *S.aureus* and *A. niger*.

Initial dose, C (µg mL ⁻¹)	Kinetic Equations	A	Half-life t _{1/2} (days)	R
<i>Escherichia coli</i> + DEP				

				0 . 9 9 7. 65 (h) 9. 17 (h) 8. 37 (h) 9. 47 (h) . 9 9 7 9
50 100 200 300	$\ln C = -0.00151t + 6.64$ $\ln C = -0.00126t + 7.24$ $\ln C = -0.00138t + 8.85$ $\ln C = -0.00122t + 10.15$	6.64 7.24 8.85 10.1 5		
<i>Staphylococcus aureus</i> + DEP				
				0 . 9 9 5. 58 (h) 6. 88 (h) 10 .0 5 (h) 7. 65 (h) . 9 9 8 6
50 100 200 300	$\ln C = -0.00207t + 6.39$ $\ln C = -0.00168t + 7.55$ $\ln C = -0.00115t + 9.54$ $\ln C = -0.00151t + 11.90$	6.39 7.55 9.54 11.9 0		
<i>Aspergillus niger</i> + DEP				
50 100 200 300	$\ln C = -0.00335t + 11.44$ $\ln C = -0.00133t + 7.15$	11.4 4 7.15 6.21	3. 45 (h)	0 . 9 9

	$\ln C = -0.00056t + 6.21$ -	-	8. 69 (h) 20 .6 3 (h) -	5 9 0 . 9 8 6 0 . 9 9 7 8 -
<i>Escherichia coli</i> + DBP				
50 100 200 300	$\ln C = -0.00275t + 7.50$ $\ln C = -0.000736t + 5.62$ $\ln C = -0.00181t + 9.96$ $\ln C = -0.000935t + 7.32$	7.50 5.62 9.96 7.32	4. 20 (h) 15 .7 0 (h) 6. 38 (h) 12 .3 6 (h)	0 . 9 9 9 7 0 . 9 9 3 0 . 9 9 8 7 0 . 9 9 7 9 -
<i>Staphylococcus aureus</i> + DBP				
50 100 200 300	$\ln C = -0.00148t + 7.73$ $\ln C = -0.00115t + 8.81$ $\ln C = -0.00044t + 8.08$ $\ln C = -0.00037t + 5.85$	7.73 8.81 8.08 5.85	7. 81 (h) 10 .0 5 (h) 1. 09 (d)	0 . 9 9 8 6 0 . 9 9 9 2 0

			1. 30 (d)	. 9 9 8 4 0 .9 9 8 6
<i>Aspergillus niger</i> + DBP				
50 100 200 300	$\ln C = -0.00272t + 9.13$ $\ln C = -0.00270t + 12.18$ $\ln C = -0.000574t + 7.88$ -	9.13 12.18 7.88 -	4. 25 (h) 4. 28 (h) 20 .2 7 (h) -	0 .9 9 6 4 0 .9 9 8 2 0 .9 9 8 3 -

Zeng *et al.* (2004). Generally, *A. niger*, the fungus was found to be the most efficient degrader of these two PAEs.

§ 4.2 Spectrophotometric Experiments

4.2.1 Spectrophotometric assays

4.2.1.1. Microbial cell surface hydrophobicity and autoaggregation of the microbes

The DEP and DBP dose used to investigate the hydrophobicity and autoaggregation ability of the microbes was 50 µg mL⁻¹. For computing microbial CSH, a quantitative method base on adherence to liquid hydrocarbon was illustrated. The ease of this technique lends itself to the rapid screening of numerous microbial samples. Three microbes were investigated for the relationship between microbial CSH and the degradation of DEP and DBP; by measuring microbial adhesion to *n*-octane (Fig. 4.4) and on their autoaggregation abilities (Fig. 4.5). There were significant differences in CSH (*E. coli*, 64.90 %; *S. aureus*, 78.25 %; and *A. niger*, 51.30 %) and the autoaggregation abilities (*E. coli*, 63.00 %; *S. aureus*, 78.25 %; and *A. niger*, 56.25 %) amongst the microbes without DEP or DPB treatment. *S. aureus* showed high (as observed by Ljungh *et al.*, 1985) while *E. coli* and *A. niger* showed moderate hydrophobicity and autoaggregation abilities in the absence of DEP or DBP treatment.

The fungus (*A. niger*) displayed lower CSH and autoaggregation than that of the bacterial microbes (*E. coli* and *S. aureus*), illustrating that remarkable differences might exist in the bacterial and fungal surface composition and structure (*i.e.*, strain- or microbe-specific characteristics).

When DEP or DBP (50 $\mu\text{g mL}^{-1}$) was added to the culture media, there were decreases in both CSH [(*E. coli*, 43.85 %; *S. aureus*, 59.40 %; and *A. niger*, 31.70 %) for DEP; (*E. coli*, 57.70 %; *S. aureus*, 65.95 %; and *A. niger*, 43.10 %) for DBP] and autoaggregation ability [(*E. coli*, 35.00 %; *S. aureus*, 58.00 %; and *A. niger*, 23.00 %) for DEP; (*E. coli*, 43.25 %; *S. aureus*, 65.00 %; and *A. niger*, 36.25 %) for DBP] for the microorganisms. When compared with the blank (0.0 $\mu\text{g mL}^{-1}$), the two moderately hydrophobic cells recorded considerable reductions in autoaggregation ability (for DEP: *E. coli*, 44.44 %; *S. aureus*, 25.88 %; and *A. niger*, 59.11 %; and for DBP: *E. coli*, 31.35 %; *S. aureus*, 16.93 %; and *A. niger*, 35.56 %) than the strongly hydrophobic cell. Similar reduction in hydrophobicity for all microbes (for DEP: *E. coli*, 32.90 %; *S. aureus*, 24.09 %; and *A. niger*, 19.64 %; and for DBP: *E. coli*, 11.09 %; *S. aureus*, 15.72 %; and *A. niger*, 15.98 %) was also observed. Such reductions made the cells less hydrophobic and less autoaggregating. These decreases are hard to explain as little is known about the effect of metabolic inhibitor compound (like DEP or DBP) on microbial cell surface hydrophobicity; however, Teo *et al.* (1998) proposed that the disappearance of cell clusters can cause inhibition of proton translocation across the microbial cell surface, resulting in reduced dehydration of cell surface and subsequently leading to decreased hydrophobicity.

This may be the likely explanation for the observed decrease in the hydrophobicity and/or adherence, thus decreasing the autoaggregation ability of the three cells. Furthermore, a possible reason for such differences could be attributed to the different compositions and origins of the microbes and the different compositions of nutrient medium; and that the surface physico-chemical characteristics of these cells are species-specific and probably decided by differences in chemical composition in the cell membrane (Christophe *et al.*, 1997). A thorough understanding of all the significant factors, which influence the whole process, is of immense desire; hence, different methods to determine hydrophobicity may lead to differences to some extent. Fig. 4.6 shows the kinetics of autoaggregation followed by the changes in the absorbance of the cultured medium in the absence and presence of DEP and DBP (50 $\mu\text{g mL}^{-1}$). No significant decrease in absorbance was observed in all the cells. The trends of CSH and autoaggregation in Figs. 4.4 and 4.5 share some similarities. Regression analysis shows a significant positive linear correlation between hydrophobicity of microbial cultures and autoaggregation abilities of the microbes as depicted in Fig. 4.7 with $R = 0.9584$ for DEP and $R = 0.9968$, which is consistent with the literature (Jain *et al.*, 2007). In general, non-parametric statistical analysis showed significant qualitative correlation between classes of hydrophobicity (*i.e.*, high, moderate and low) and antibiotic resistance (IC_{50} values) and from the regression analysis (Fig. 4.8) some kinds of relationships were also found: a non significant positive linear one ($R = 0.3839$; DEP) and, of CSH and IC_{50} [which contradicted the finding of Tahmourespour *et al.* (2008) but supportive of those of Pan *et al.* (2006) and Kuntiya *et al.* (2005)]. It was found that there is somehow negative relationship (Fig. 4.9) between CSH and IC_{50} , *i.e.*, the smaller the CSH, the larger the IC_{50} for the cells, indicating

that decreasing the CSH will lower down the toxicity of DBP to the microbes which is in agreement with Tahmourespour *et al.* (2008). The correlation between CSH and the DBP microbial degradation was also found to be negatively related. Similar results were reported by Al-Tahhan *et al.* (2000) and Prabhu and Phale (2003) that as the cell surface becomes more hydrophobic, the association of the cell with the hydrophobic substrate becomes stronger, resulting in an increase in the degradation rate.

The possible reasons for the observed trends in hydrophobicity and autoaggregation ability versus DEP or DBP biodegradation are that: (a) the presence of inhibitors enhances exopolysaccharide (EPS) production (Raihan *et al.*, 1992; Singh and Fett, 1995) even though the mechanism has not been fully understood, which may, however, account for a decreased hydrophobicity (Caccavo *et al.*, 1997) when the EPS are predominantly neutral or hydrophilic; (b) increase in the age of the cell leads to higher EPS production (Fattom and Shilo, 1984); and (c) nutrient starvation in the culture may (Wrangstadh *et al.*, 1986) also cause EPS production, thereby reducing the hydrophobicity. Additionally, Medrzycka (1991) and Busscher *et al.* (1995) reported that during surface hydrophobicity assay, the hydrocarbons used are negatively charged which may cause repulsive (for Gram-negative cells) or

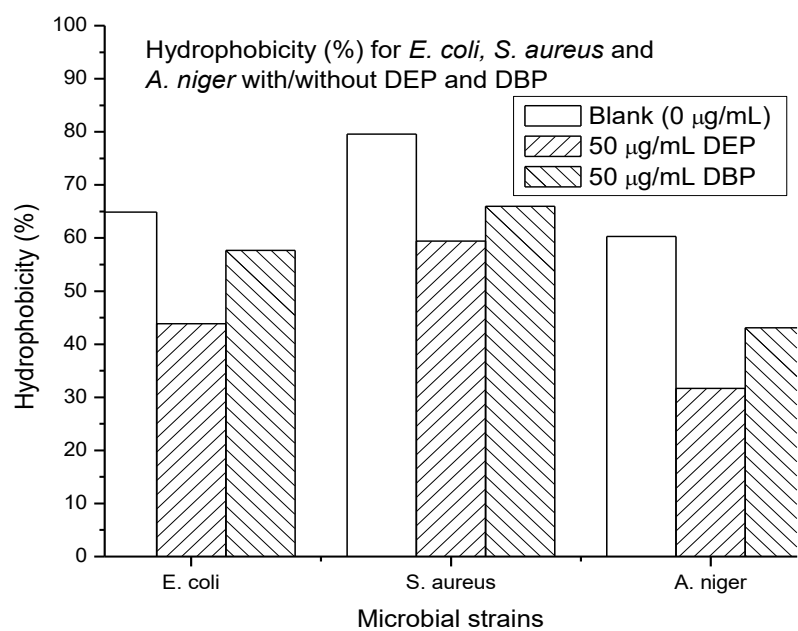


Fig. 4.4. Surface Hydrophobicity of the three microbial strains grown in their respective medium only or a medium containing DEP and DBP.

attractive (for Gram-positive cells) electrostatic interactions; thus, interfere with adherence of cells to hydrocarbons.

4.2.1.2 The Effects of the PAEs on the Turbidity (OD₆₆₀) of culture medium and cell dry weights (Biomass).

The growths of the three microbes were monitored in their respective culture media in the absence or presence of the PAEs at various dosages and the corresponding changes in the biomass dry weights and turbidities were

recorded and respectively plotted on Fig. 4.10 and Fig. 4.11. The relationship between biomass and turbidity was literally observed to be linear. The changes in turbidity and biomass (at the log phase), as a result of PAE addition, with incubation time were found to be exponential [(hence, obeying the thermokinetic equation, Equation (8), (Hall and Hawkins, 1975))] and dose-dependent; and depict the phases of growth.

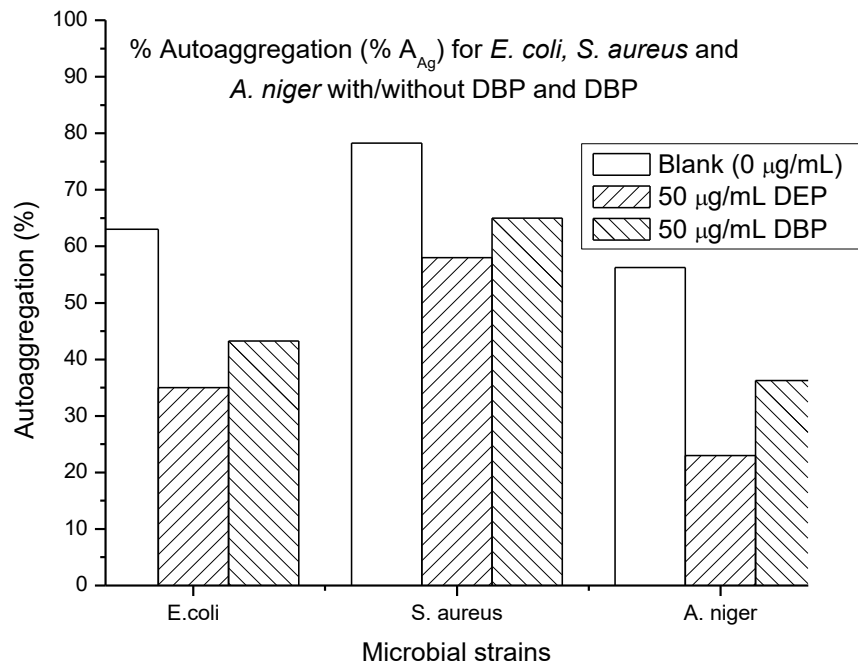


Fig. 4.5. Autoaggregation ability of the three microbial strains grown in their respective medium only or a medium containing DEP and DBP.

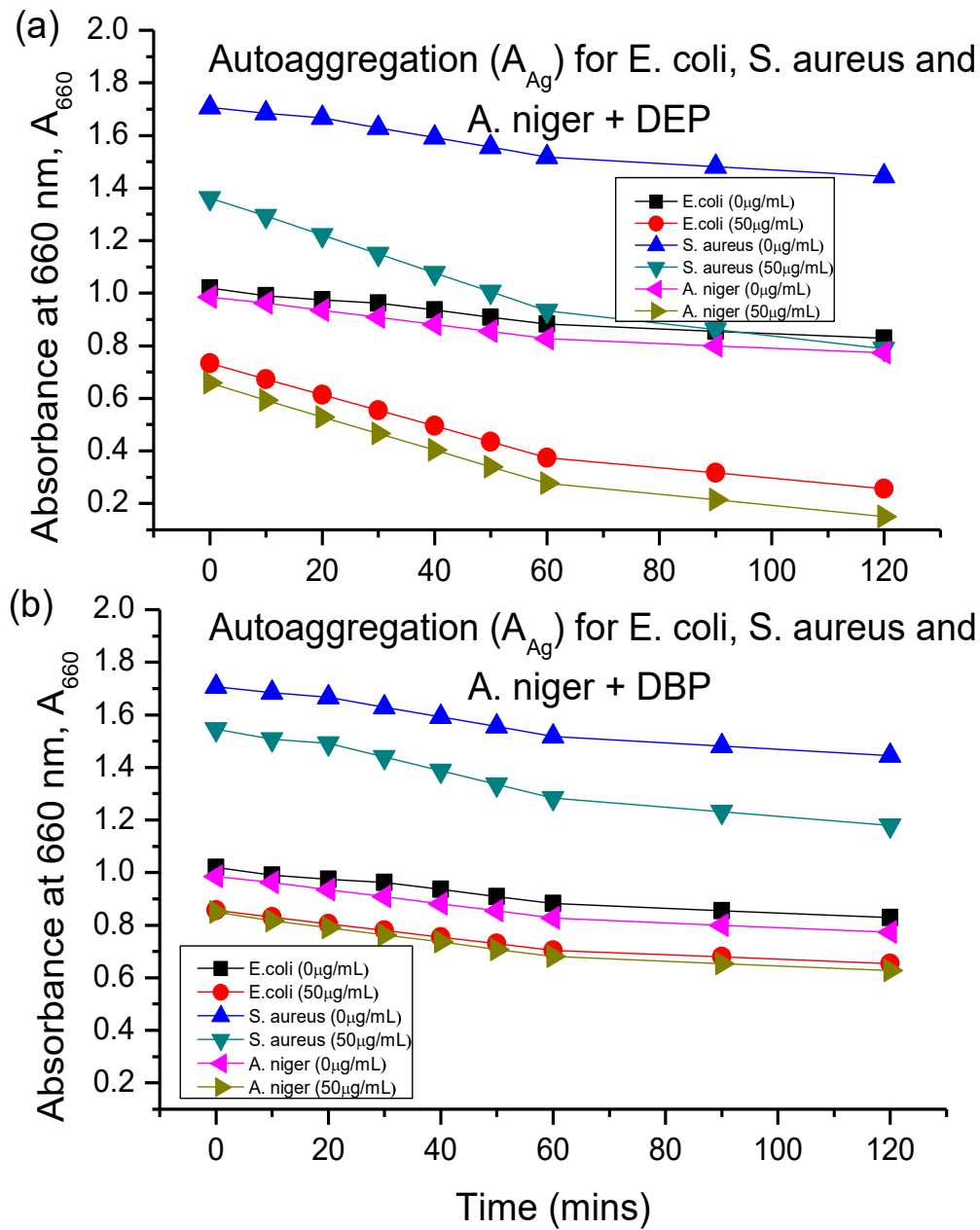


Fig. 4.6. Autoaggregation kinetics of the three microbial strains to n-octane grown in their respective medium only or a medium containing (a) DEP and (b) DBP.

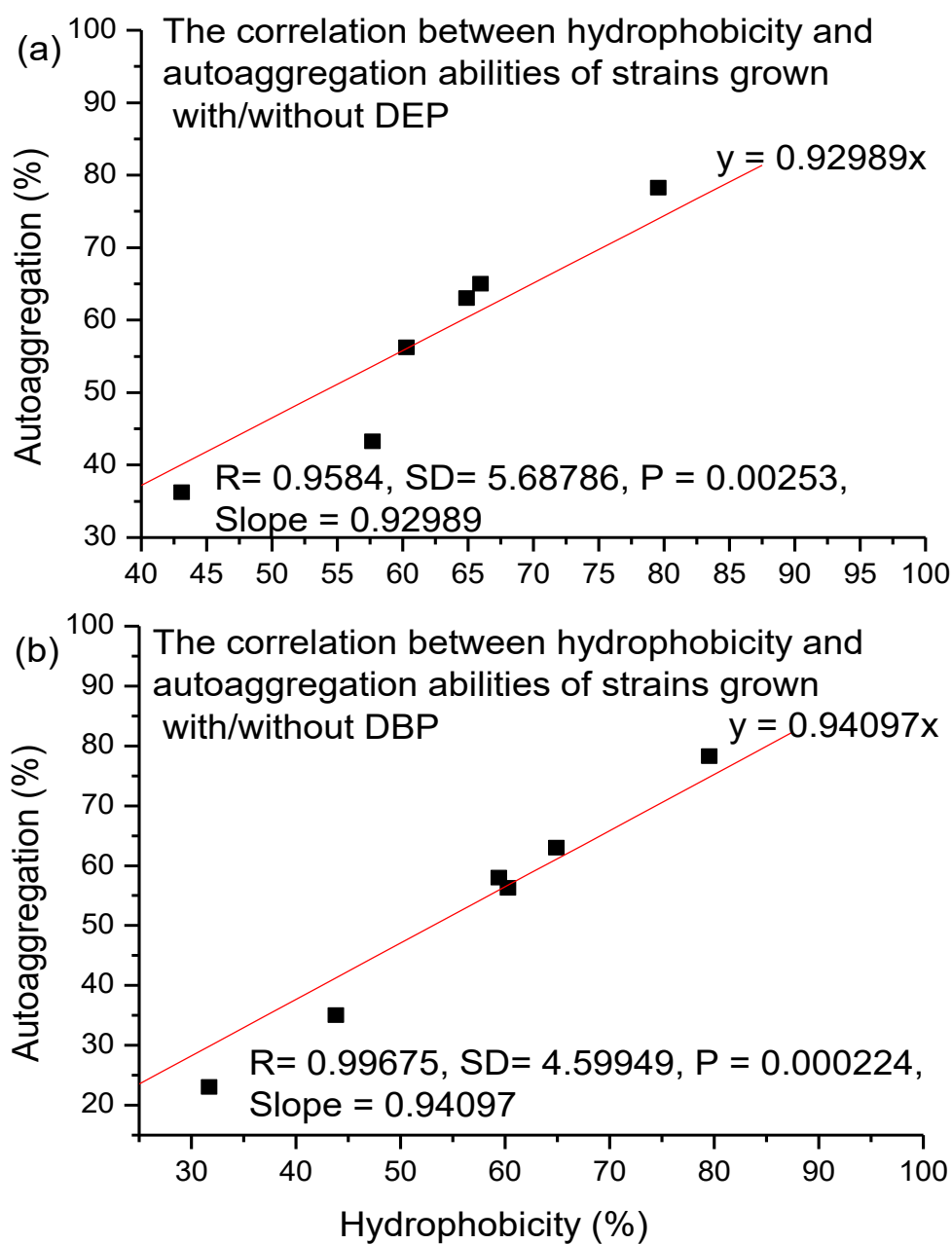


Fig. 4.7. The correlation between autoaggregation ability and the hydrophobicity of the microbes in medium containing (a) DEP and (b) DBP.

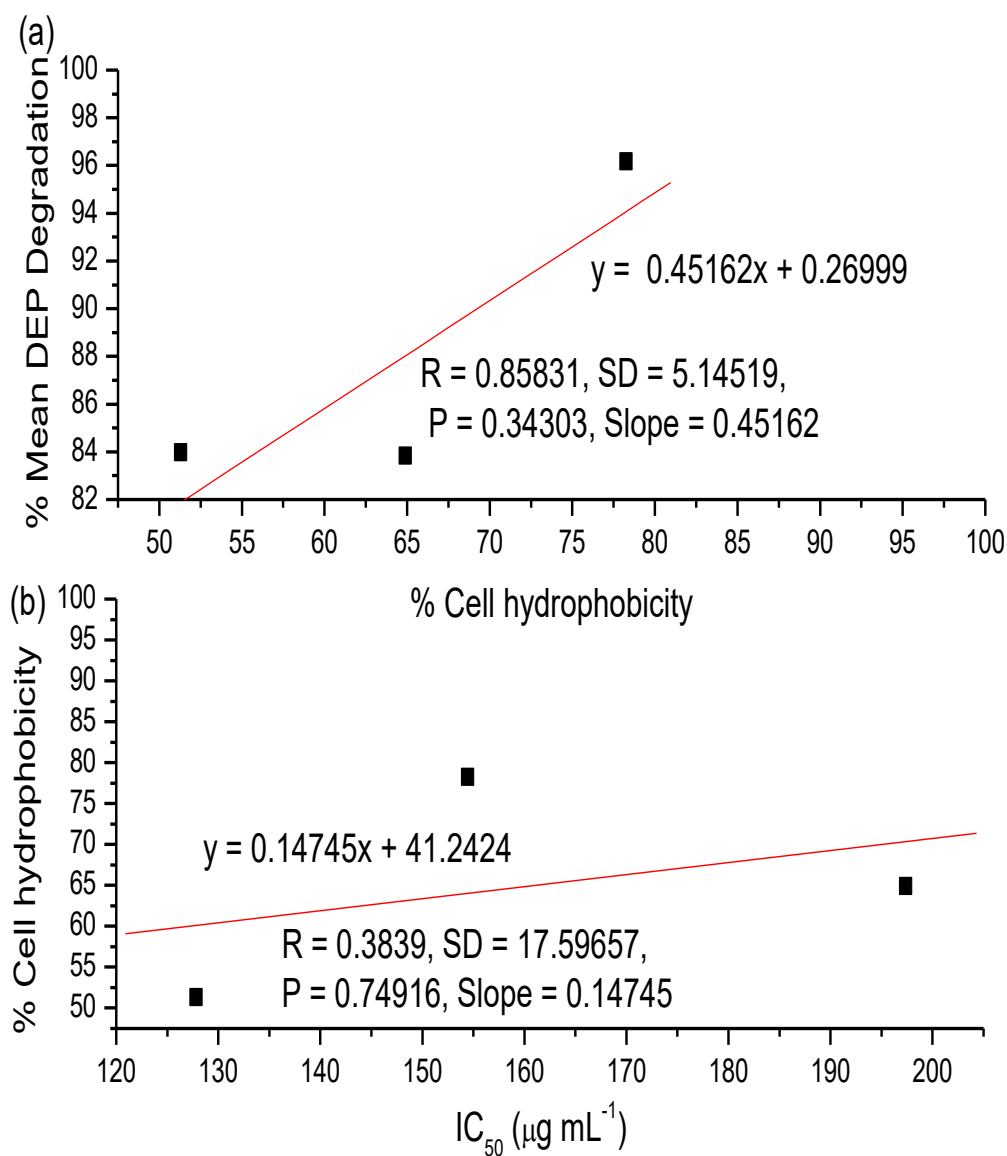


Fig. 4.8. The correlation between the microbial cell surface hydrophobicity and (a) mean DEP degradation efficiency and, (b) inhibition (IC_{50}).

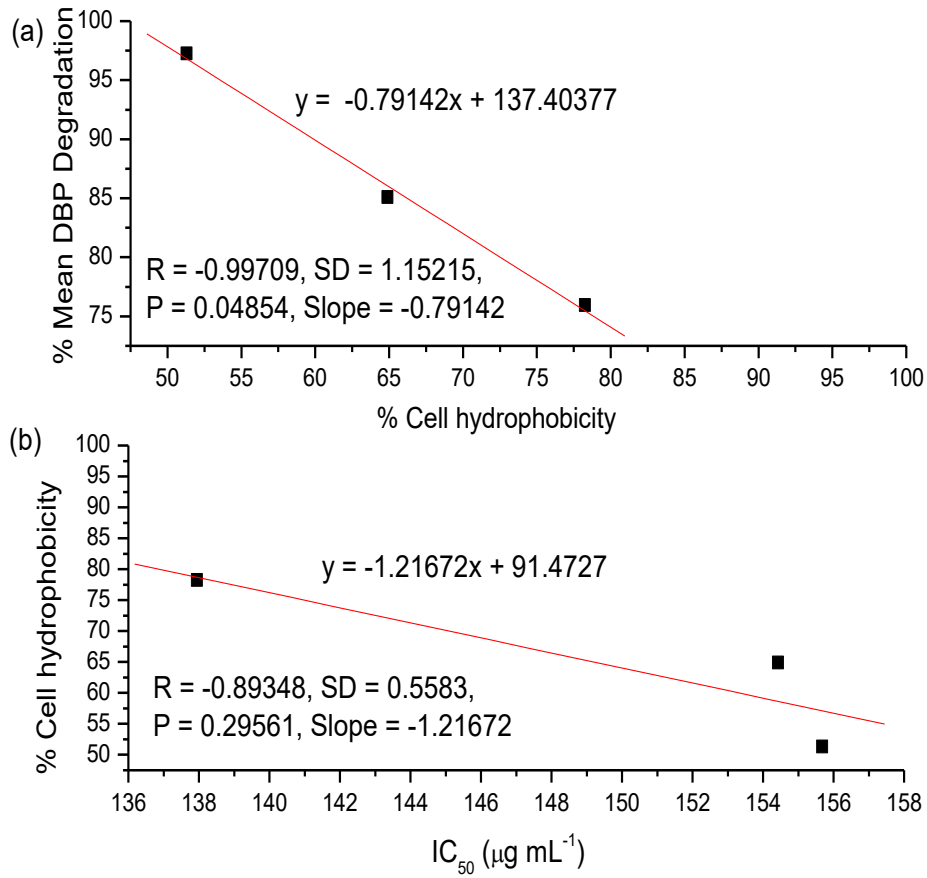


Fig. 4.9. The correlation between the microbial cell surface hydrophobicity and (a) mean DBP degradation efficiency and, (b) inhibition (IC_{50}).

It can be seen from Fig. 4.10 that $200 \mu g mL^{-1}$ each of DEP and DBP can be used by all the test microbes to enhance their growth. However, the metabolizing rates were different. *A. niger* appeared to be the most effective of the three microbes for DEP and DBP degradation, respectively, which degradations took place faster than the other two microbes under the same experimental conditions. The experiments lasted for 100 and 120 hours to respectively degrade $200 \mu g mL^{-1}$ each of DEP and DBP, and that degradation was accompanied by an increase in biomass concentration (Fig. 4.10). The log phase of biomass curves also obeys Equation (8), and the growth rate constant $k_{biomass}$.

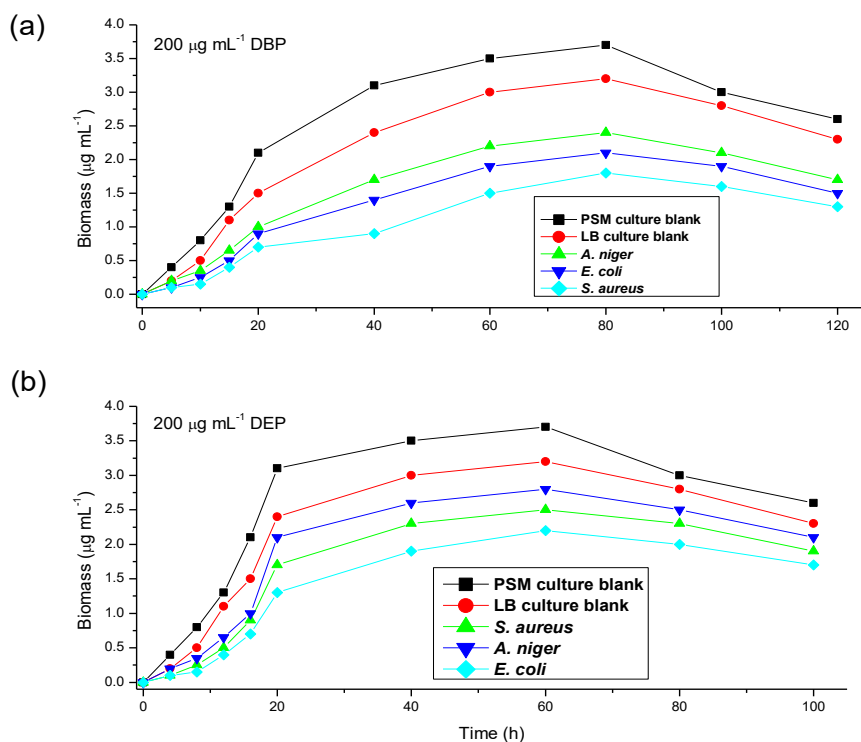


Fig. 4.10. Biomass dry weight/Growth curves of the three microbes influenced by 200 $\mu\text{g mL}^{-1}$ a) DBP and b) DEP at 28 °C.

Fig. 4.11 demonstrated that the lag phase duration of microbial cell growth and DEP and DBP degradations prolonged with increase of initial DEP and DBP concentrations, that is to say, the microorganisms exhibited extended lag time at higher initial DEP and DBP concentrations. Although 300 $\mu\text{g mL}^{-1}$ each of these compounds can also be degraded completely, the times needed for degradation were

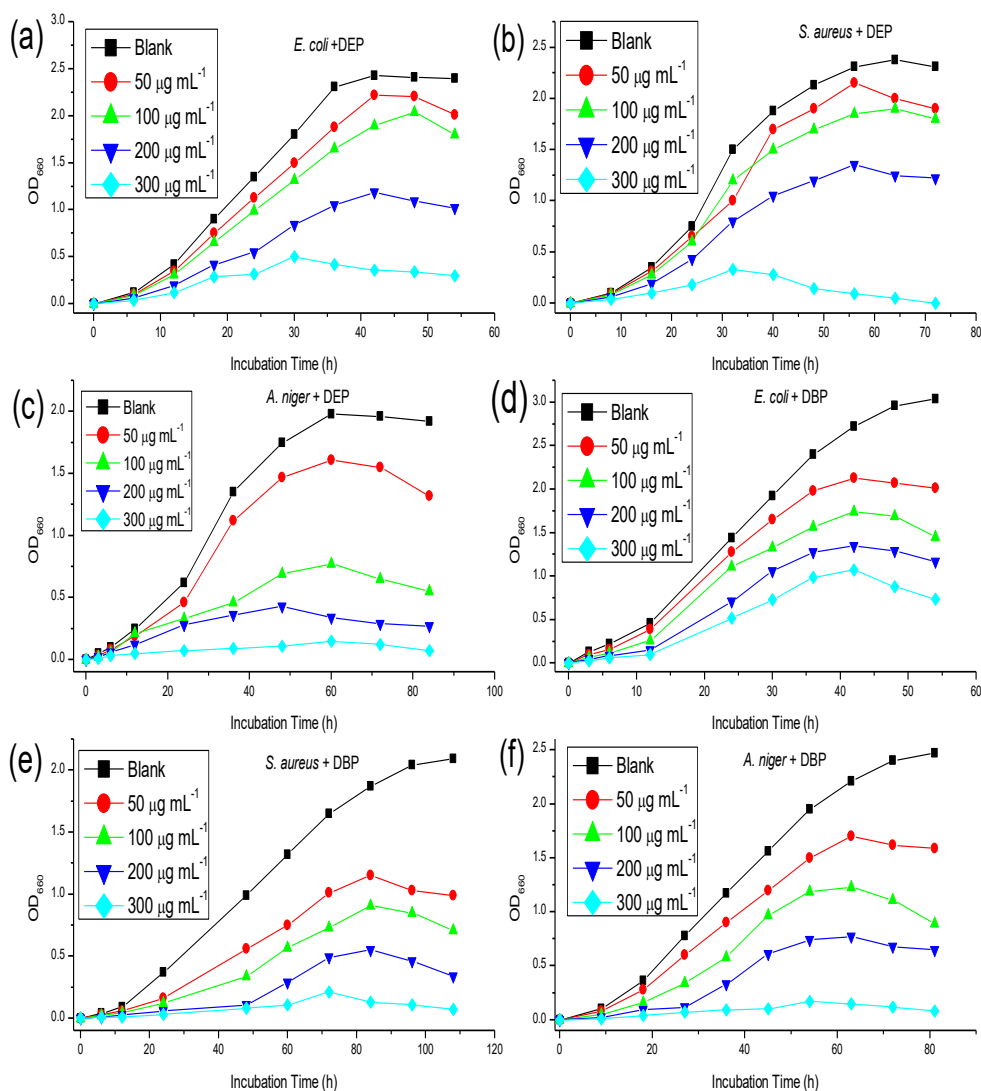


Fig. 4.11. Turbidity changes at 660nm (OD_{660}) with incubation time of the three microbes in the absence (blank) and presence of the various concentrations of DEP [(a), (b), (c)]; and DBP [(d), (e), (f)]

longer than the ones needed for lower initial concentrations of DEP or DBP for *S. aureus*, *A. niger* and *E. coli*, respectively. It's noteworthy that all three microbes completely degraded both PAEs within the same time period (i.e. about 84 to 100 h). However, once the degradation started, its degradation rate was not inhibited.

Fig. 4.11 also demonstrated that the biomass concentration increased with increasing initial DEP/DBP concentration(s). In addition, it was found that the microorganism continued to grow for a certain time after DEP/DBP was consumed, which indicated that intermediates accumulated during the primary degradation of DEP/DBP, could subsequently be used for microbial growth. DEP/DBP biodegradation follows the general pathway, Equation (9) (Kurane *et al.* 1978). The proposed degradative pathway involves sequential cleavage of the ester bond to yield the phthalate monoester and then phthalic acid which is further metabolized and enters the tricarboxylic acid (TCA) cycle to produce carbon dioxide and water.

Despite the effective degradation rate of these two PAEs, dose-dependent growth inhibition rates were also obtained from OD_{660} via Equation (5b) by interpolation regression analysis using OriginPro 8.0. The mean IC_{50}

values for DEP and DBP obtained from these growth inhibition assays are shown in Table 4.5 and Appendices 12 (a-f). The IC_{50} values (*E. coli*, 190.91 $\mu\text{g mL}^{-1}$; *S. aureus*, 193.08 $\mu\text{g mL}^{-1}$; and *A. niger*, 154.28 $\mu\text{g mL}^{-1}$) in Table 4.5 indicate that *A. niger* and *S. aureus* are the most sensitive and most tolerant microbes to DEP, respectively; and those (*E. coli*, 194.34 $\mu\text{g mL}^{-1}$; *S. aureus*, 142.61 $\mu\text{g mL}^{-1}$; and *A. niger*, 138.04 $\mu\text{g mL}^{-1}$) in Table 4.5 indicate that *A. niger* and *E. coli* are the most sensitive and most tolerant microbes to DBP, respectively. When compared to the mean IC_{50} values obtained calorimetrically, similar trends were found with higher values obtained from the OD_{660} assay. This can be explained by the fact that calorimetric experiments were performed largely under anaerobic conditions and the OD_{660} assays were done under aerobic conditions, as the culture media were well shaken up leading to more oxygen supply to the system, better microbial growth and longer generation times. Hence making the mean IC_{50} values obtained from the two methods different. From Table 4.5, it was also observed that the higher the PAE concentration, the higher the percent inhibitory ratio (%I), and increased toxic potentiality.

§4.3 Chromatographic Analyses

4.3.1 DEP and DBP degradation Time courses and cell growth by different microbes.

The DEP and DBP biodegradations using different initial concentrations were studied with free and pure cells of *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger* and the results were plotted in Fig. 4.12. The time courses of the two PAEs biodegradation rates and tolerance by these microbes at the different initial concentrations of DEP and DBP were shown in Fig. 4.12. This figure showed that DEP and DBP can be rapidly and completely degraded within 84 h (i.e., 3.5 days) and 120 h (i.e., 5 days) maximum, respectively. However, the degradation rates of DBP were slow. These degradation trends were also observed in the microcalorimetric assays. The biodegradation rates of these two PAEs appeared to be related to the length of the alkyl side chains, influencing the length of the alkyl group of the di-ester, the biodegradability (Jianlong *et al*, 1996). As it is evident from Fig. 4.12, the biodegradation rates of the two PAEs were different. The shorter-chain DEP was degraded at a higher rate than the longer-chain DBP. The three cultures were able to degrade actively up to 300 $\mu\text{g mL}^{-1}$ dose of DEP and DBP, however, at slower rates. The degradations of these two tested phthalates conform to the first-order reaction model.

The tests for DEP and DBP biodegradation were carried out using GC measurements. In order to eliminate the possibility of PAEs decomposition by chemical reactions such as hydrolysis and oxidation, their degradations were examined using sterile controls (for both PAEs) of uninoculated media incubated/autoclaved with the other flasks. No degradation was observed which suggested that abiotic loss of PAEs can be neglected during this study. The time courses of 0 - 300 $\mu\text{g mL}^{-1}$ each of DEP and DBP degradation by different microbes were shown in Fig. 4.12.

The order of the biodegradability of the two PAEs by these microbes was observed to be in this order: DEP (*S. aureus*) > DBP (*A. niger*) > DEP (*A. niger*) > DEP (*E. coli*) > DBP (*E. coli*) > DBP (*S. aureus*). This trend was observed in Sections

4.1.3 and 4.1.4, and also confirmed the observation made by other researchers, including Zeng *et al.* (2004). Generally, *A. niger*, the fungus was found to be the most efficient degrader of these two PAEs.

4.3.2 Identification of metabolites of PAE biodegradations

The metabolites of PAE biodegradation (Table 4.4) were identified by the Thin-layer chromatography (TLC). Based on R_f , the intermediates released into the medium appear to be monoethyl phthalate (MEP), monobutyl phthalate (MBP) and phthalic acid (Table 4.4). Benckiser and Ottow (1982) reported that under anaerobic conditions, DBP (or DEP) is hydrolyzed via monobutyl phthalate, MBP (or monoethyl phthalate, MEP) to PA. Protocatechuate, a key intermediate during aerobic degradation of DBP via MBP and PA by numerous microorganisms (Evans, 1997), was not detected in this study. These results suggested that MEP (from DEP degradation) MBP (from DBP degradation) and PA were the ultimate products of DEP and DBP hydrolysis. The R_f values are strongly dependent upon the nature of the adsorbent and solvent. Therefore, experimental R_f values and literature values do not often agree

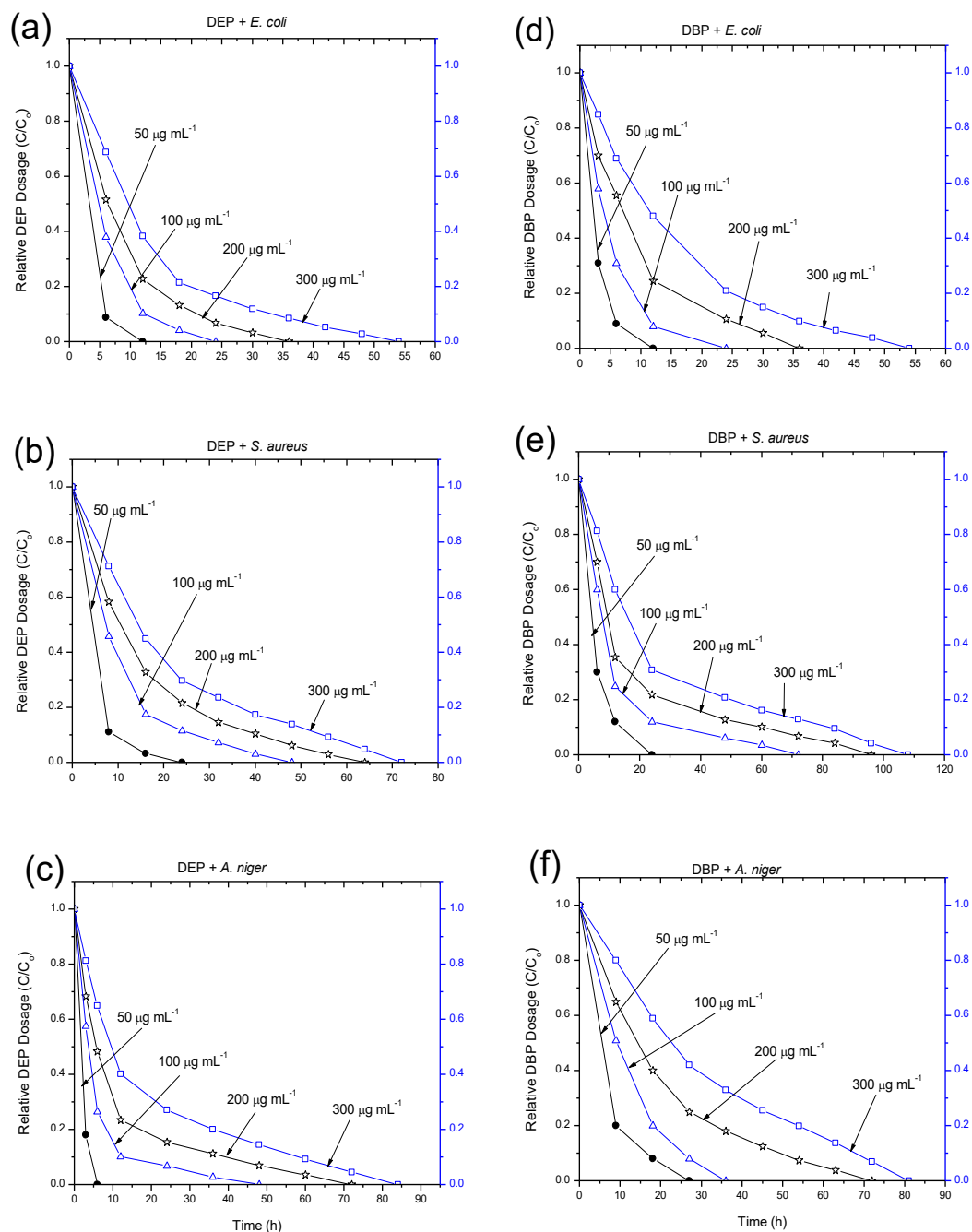


Fig. 4.12. Degradations by the three strains at various initial concentrations of DEP [(a), (b), (c)] and DBP [(d), (e), and (f)] by the GC measurements.

very well. In order to determine whether an unknown substance is the same as a substance of known structure, it is necessary to run the two substances side by side in the same chromatogram, preferably at the same concentration.

Table 4.4. Metabolites (degradation products) of DEP and DBP degradations under denitrifying

conditions^a.

Compounds	R_f value
<i>Metabolites</i>	
X	0.35
Y	0.08
Z	0.68
<i>References</i>	
DBP	0.76
MBP	0.36
DEP	0.89
MEP	0.68
PA	0.08
Protocatechuic acid	0.15

^aThe solvent is benzene:acetic acid (9:1).

§4.4 Chapter Summary

This research disclosed that there are significant correlations between microbial cell surface hydrophobicity and autoaggregation ability, biodegradability, IC_{50} values. This finding suggests the significance of hydrophobic interactions in the attachment of these cultures to chemical compounds and thus DEP/DBP biodegradability by these microbes.

The results obtained in the present study suggest that up to 300 $\mu\text{g mL}^{-1}$ DEP or DBP can be completely degraded within 108 h by isolated microbes. However, the degradation rates were different. The main metabolites of DEP and DBP degradations appeared to be monoethyl phthalate, monobutyl phthalate and phthalic acid.

Table 4.5. Means of the calculated % Inhibition and IC_{50} ($\mu\text{g mL}^{-1}$) values from OD_{660} values.

Test	0 μ g m L - 1	5 0 μ g m L - 1	1 0 0 μ g m L - 1	2 0 0 μ g m L -1	3 0 0 μ g m L - 1	<i>IC</i> ₅₀ (μg mL ⁻¹)
<i>E. coli</i> + DEP	0 . 0 0 %	1 4 . 9 9 %	2 4 . 6 8 %	5 4 . 0 1 %	7 7 . 2 0 %	190.9 1
<i>S. aureus</i> + DEP	0 . 0 0 %	1 4 . 3 5 %	2 0 . 2 8 %	4 4 . 3 4 %	8 4 . 7 7 %	193.0 8
<i>A. niger</i> + DEP	0 . 0 0 %	2 3 . 7 5 %	5 4 . 2 9 %	6 7 . 7 3 %	8 7 . 6 0 %	154.2 8
<i>E. coli</i> + DBP	0 . 0 0 %	2 1 . 5 4 %	3 9 . 3 9 %	5 5 . 3 9 %	6 8 . 7 9 %	194.3 4
<i>S. aureus</i> + DBP	0 . 0	4 2 .	5 7 .	7 7 .	9 0 .	142.6 1

	0 %	3 5 %	9 5 %	4 1 %	1 1 %	
<i>A. niger</i> + DBP	0 . 0 0 %	3 5 . 6 3 %	6 3 . 9 7 %	7 3 . 6 8 %	9 6 . 7 6 %	138.0 4

Comparing the experimental results of calorimetry (under anaerobic conditions), spectrophotometric and chromatographic determinations, little differences can be seen. This is owed to the distinction between and among the methods. These microbes were intact in the stainless-steel ampoules during the determinations by use of calorimetry; on the other hand, shake flasks experiments inevitably influenced the growth of these facultative microbes. During the operation, the culture media needed shaking up to ensure that the biomass was well proportioned; this led to more oxygen that was utilized by the cells. Under this condition, the cells grew well and had longer generation times. Hence the IC_{50} values of the toxic actions of these PAEs on the cells were different too.

The experimental results of calorimetry and chromatography also demonstrated that DEP and DBP were rapidly degraded, about 90% within 2 and 3 days and completely degraded within 3.5 and 4.5 days, respectively. It can be concluded that DEP and DBP can be degraded although at different rates. These biodegradation rates of these two PAEs appeared to be related to the length of the alkyl side chains; the lengths of the alkyl side groups of the diesters influence the biodegradabilities. As it is evident in Figs. 4.1, 4.2, 4.10 and 4.11; Tables 4.1 and 4.2; and the Appendices 12 (a) – (g), the rate of degradation of the two PAEs were different. The shorter-chain DEP was degraded at a higher rate than the longer-chain DBP.

CHAPTER FIVE: SUMMARY, GENERAL CONCLUSIONS AND RECOMMENDATIONS

Summary, conclusions (arising from the research results) and recommendations form Chapter Five. This part lists

the main results/findings of the research and makes conclusions and perspectives for future research within the field.

§5.1 Summary

PAEs are synthesized in massive amounts to produce various plastics and have become ubiquitous/widespread in environments and have been found in the pedosphere (soils, landfills and leachates), plants, hydrosphere (i.e. natural waters, fresh and ocean waters, groundwaters), the atmosphere, and human and other living organisms' fatty tissues (compounds are lipophilic/lipophilic; i.e. fat-soluble) as a result of the production, usage, and disposal of plastics. Such worrying findings have aroused interest in the biodegradation of these compounds in soils and waters. Six of the 20 PAEs produced for commercial purposes have been considered as priority pollutants by the United States Environmental Protection Agency and People's Republic of China. These compounds are also included in the "priority list of substances for further evaluation of their role in endocrine disruption" by the European Commission. Among these six PAEs, DEP and DBP are amongst the most frequently identified PAEs in environmental matrices.

The main aim of this study was to evaluate the microbial degradability of two potentially toxic PAEs (i.e., DEP and DBP) by two bacteria (*Escherichia coli* and *Staphylococcus aureus*) and a fungus (*Aspergillus niger*) using microcalorimetry by monitoring their growth on media amended with/without DEP or DBP. To achieve this aim, the following specific objectives were carried out:

- (a) **January – April 2008:** Investigated the target organisms for their cell surface properties, including cell autoaggregation ability, hydrophobicity, etc, as these properties determine their survival in hazardous situations by controlling the process of association between the cells and other surfaces, thereby, playing an important role in degradation of hydrophobic substrates;
- (b) **June – December 2008:** Used the TAM III microcalorimeter (as the main research tool/instrument) to determine the respective degradation abilities of these two organic compounds by these test microbes; and kinetic equations of the reactions taking place in the microcalorimeter (at a constant temperature of 28 °C and heater pulse power of 120 µW) were also developed.
- (c) **September – November 2009:** Also to serve as confirmatory tests, a gas chromatography - GC was used to evaluate the degradation efficiencies of each of these PAEs by these microorganisms; and identified the residues (or metabolites) of these PAE degradations using thin-layer chromatography (TLC);
- (d) **January – April 2009:** Validated the results got from the above experiments, traditional physico-microbial tests, like shake flask experiments, were carried out to determine the biomass and optical density (OD)/turbidity using spectrophotometry.

The research results showed that; for DEP, there are significant correlations between microbial cell surface hydrophobicity (CSH) and autoaggregation ability, biodegradability, IC_{50} values. These findings suggest the importance of hydrophobic interactions in the attachment of these cultures to chemical compounds. It was very clear that hydrophobicity had some kind of relation to autoaggregation ability and thus DEP/DBP biodegradability for these species. So the substantiation of correlation between surface hydrophobicity and autoaggregation, vis-à-vis DEP or DBP biodegradation and inhibitory effect can allow us to perform direct screening of promising adherent microbial cells with the efficacy for useful microorganisms without using complicated and protracted methods. This study also provides information on understanding how these microbes work very well on the biodegradation of DEP and DBP, thereby, identifying them as potential degraders of DEP and DBP and then their possible use in the DEP and DBP clean-up in the environment.

In this study, microcalorimetric method was employed as the main tool to study the biodegradation of two PAEs of the same concentration range ($50 - 300 \mu\text{g mL}^{-1}$) by *E. coli*, *S. aureus* and *A. niger*. From the results of this study, it was observed that these species gave out more heat in the presence of PAE than in its absence, but they (microbes) were inhibited when the PAE concentration reached $300 \mu\text{g mL}^{-1}$. The results also showed that PAEs have biphasic effects on the metabolism of *E. coli*, *S. aureus* and *A. niger*, i.e. at lower concentrations they (PAEs) can stimulate the growth of these microbes, while at higher concentrations PAE can inhibit their growth. The results obtained from *E. coli*, *S. aureus* and *A. niger* are in agreement with those gained from other microorganisms by other microbiological methods. Since *E. coli*, *S. aureus* and *A. niger* are regarded as typical true microbes and have become some of the best understood organisms at the molecular level, it is anticipated that microcalorimetry could provide new and, perhaps, more comprehensive information about biological effects of other PAEs on other microorganisms and their consequent degradation by these (other) organisms. The results obtained in the present study suggest that up to $300 \mu\text{g mL}^{-1}$ DEP or DBP can be completely degraded within 120 h by these target microbes. However, the degradation rates were different. The main metabolites of DEP and DBP degradations, identified by the Thin-layer chromatography (TLC) based on their R_f values, appeared to be monoethyl phthalate (MEP), monobutyl phthalate (MBP) and phthalic acid (PA). Protocatechuate, a key intermediate during aerobic degradation of DBP via MBP and PA by numerous microorganisms, was not detected in this study.

Microcalorimetry can be a useful and accurate supplement in the study of microbial inhibition (toxicity), degradation of chemical compounds by determining valuable physico- and bio-chemical parameters including IC_{50} and determination of the sub-MIC values. This study pointed out that results obtained by microcalorimetry were in agreement with those from turbidity (OD_{660})/biomass dry weight assays and chromatographic methods in this present work and other parallel tests involving other microorganisms and PAEs. These methods indicated that DEP and DBP have stimulatory effects in certain concentration ranges and that there was good agreement between the traditional microbiological methods and the microcalorimetric method. The obvious advantage of the automated microcalorimetric method over the former methods is that it allows multiple comparisons of

different PAEs–microbe combinations at the same time with high reproducibility and less errors.

The microbes employed in the experiments were all capable of degrading DEP, and DBP with degradation rates were very high and degradation capacities were also very efficient with microcalorimetry. The biodegradability of the PAEs by these microbes appeared to be related to the length of the alkyl side chains. Relatively, the shorter-chain DEP was degraded at higher rate than the longer-chain DBP. It's observed that as the octanol/water partition coefficient (k_{ow}) increased and the water solubility decreased, the biodegradability of PAEs by these microbes declined. The order of the biodegradability of the two PAEs by these microbes was DEP (*S. aureus*) > DBP (*A. niger*) > DEP (*A. niger*) > DEP (*E. coli*) > DBP (*E. coli*) > DBP (*S. aureus*). This trend was also observed in Section 4.1.3 and confirmed the observation made by other researchers, including Zeng *et al.* (2004). Generally, *A. niger*, the fungus was found to be the most efficient degrader of these two PAEs.

§5.2 General Conclusions

This study has revealed PAEs biodegradability potentials of *E. coli*, *S. aureus* and *A. niger*; organisms that have been widely considered as causing diseases. The study has also served to highlight the relationship between PAE biodegradation ability and microbial cell surface properties, including CSH, cell surface charge and autoaggregation ability. From the findings of the study, the following are drawn as the main conclusions:

The fungus (*A. niger*) displayed lower CSH and autoaggregation than that of the bacterial microbes (*E. coli* and *S. aureus*), illustrating that remarkable differences might exist in the bacterial and fungal surface composition and structure (*i.e.*, strain- or cell-specific characteristics). When DEP or DBP ($50 \mu\text{g mL}^{-1}$) was added to the culture media, there were decreases in both CSH and autoaggregation ability for the microorganisms. When compared with the blank ($0.0 \mu\text{g mL}^{-1}$), the two moderately hydrophobic cells recorded considerable reductions in autoaggregation ability than the strongly hydrophobic cell. Similar reduction in hydrophobicity for all microbes was also observed. Regression analysis shows a significant positive linear correlation between hydrophobicity of microbial cultures and autoaggregation abilities of the microbes with $R = 0.9584$ for DEP and $R = 0.9968$, which is consistent with other literatures.

From the microcalorimetric study, it was deduced that *S. aureus* [mean degradation efficiency (δ) = 96.18 %] is the most efficient DEP degrader and *E. coli* (mean δ = 83.83 %) the least; and that *A. niger* (mean δ = 97.27 %) is the most efficient DBP degrader and *S. aureus* (mean δ = 75.95 %) the least. Also the IC_{50} values (*E. coli*, $197.37 \mu\text{g mL}^{-1}$; *S. aureus*, $154.43 \mu\text{g mL}^{-1}$; and *A. niger*, $127.84 \mu\text{g mL}^{-1}$) indicate that *A. niger* and *E. coli* are the most sensitive and most tolerant microbes to DEP, respectively; and those (*E. coli*, $154.43 \mu\text{g mL}^{-1}$; *S. aureus*, $137.95 \mu\text{g mL}^{-1}$; and *A. niger*, $155.68 \mu\text{g mL}^{-1}$) indicate that *A. niger* and *S. aureus* are the most tolerant and most sensitive microbes to DBP, respectively.

It was observed that the growth rate constants decrease with the increasing concentrations of PAEs. When the

concentrations of PAEs reached $300 \mu\text{g mL}^{-1}$, the PAEs exerted complete inhibitory effects on the growth of the microbes, in this case no thermal effects can be observed in the power–time curve during the experimental time. The biodegradability of the PAEs by these microbes appeared to be related to the length of the alkyl side chains. Relatively, the shorter-chain DEP was degraded at higher rate than the longer-chain DBP.

The relationship between biomass and turbidity was observed to be linear. The changes in turbidity and biomass (log phase), as a result of PAE addition, with incubation time were found to be exponential [(hence, obeying the thermokinetic equation] and dose-dependent; and depict the phases of growth. Research data also demonstrated that the biomass concentration increased with increasing initial DEP or DBP concentration; hence they are regarded as carbon and energy sources. In addition, it was found that the microorganism continued to grow for a certain time after DEP or DBP was consumed, which indicated that intermediates accumulated during the primary degradation of DEP or DBP, could subsequently be used for microbial growth. DBP biodegradation follows the general pathway. They (PAEs) were also found to have some inhibitory effects, especially at higher concentrations.

Research data from the GC (Hewlett-Packard Model 5890; J.W., USA) analyses showed that DEP and DBP were rapidly and completely degraded within 84 h (i.e., 3.5 days) and 108 h (i.e., 4.5 days) maximum, respectively. However, the degradation rates of DBP were slow. These degradation trends were also observed in the microcalorimetric assays and the spectrophotometric methods. The shorter-chain DEP was degraded at a higher rate than the longer-chain DBP. The three cultures were able to degrade actively up to $300 \mu\text{g mL}^{-1}$ dose of DEP and DBP, however, at slower rates. The degradations of these two phthalates conform to the first-order reaction model.

Based on the R_f values, the intermediates released into the medium were identified to be monoethyl phthalate (MEP), monobutyl phthalate (MBP) and phthalic acid (PA). Protocatechuic acid, a key intermediate during aerobic degradation of DBP via MBP and PA by numerous microorganisms, was not detected in this study. These results suggested that MEP (from DEP degradation) MBP (from DBP degradation) and PA were the ultimate products of DEP and DBP hydrolysis. PA is metabolized via protocatechuic acid by 3,4-dioxygenative cleavage. Since the tested organisms can use ethanol as a sole carbon source, the entire molecule seems to be mineralized. In mammalian metabolism, also, only a single hydrolysis with accumulation of monoester of phthalate is performed (Albro *et al.*, 1973). It shows that formation of monoester is the most common reaction in the mineralization of phthalate esters.

Generally, this study determined significant data on the biodegradation of DEP and DBP by *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger* through microcalorimetry. The result may provide a useful beginning for the modeling program of PAE bioremediation in the environment. The results may also be useful in the formulation of pollution control and bioremediation policies as well as in the exploration of ways for protecting human and environmental health and can offer vital information for researches in the field of

microbiology. In the future, similar conditions can be used to further define operating parameters for PAE bioremediation in soil, sediment, air, etc, and to monitor the microbial dynamics involved in the anaerobic degradation of PAEs.

The three microbes, widely regarded as pathogens, are highly efficient in the degradation of these two PAEs. Faster and more efficient biodegradation rates were achieved from the microcalorimetric method as compared to those obtained from the chromatographic method of this study and previous studies by other researchers using methods other than microcalorimetry. The biodegradation of organic pollutants by these pure microbes is a very versatile approach. This technique can be suitably used in detoxification of different pollutants/contaminants for long periods. These microbes are efficient strain capable of degrading PAEs completely. Hence there is a great potential for the use of these cells for the treatment of industrial effluents containing PAEs.

On the other hand, the results of this study need to be interpreted with some caution. All the sampling followed the procedures obtained at the Key Laboratory of Biogeology and Environmental Geology of Chinese Ministry of Education, China University of Geosciences, Wuhan, People’s Republic of China.

§5.3 Recommendations

In line with the inferences and limitations of the present study it is largely evident that further researches are needed to compliment this present study, including:

1. Microcalorimetric measurements have generally been made using a closed ampoule method with no means of supply of O₂ which causes a decrease in the available O₂ with a corresponding CO₂ enrichment. Consequently the environmental conditions change inside the closed ampoule but the problem can be overcome in long-term studies using a microcalorimetric perfusion vessel to measure microbial activity. Aerated ampoules/vessels microcalorimeter to be used in future experiments, as in the presence of enough air (O₂), the rates and efficiencies of PAEs degradation are higher than in anaerobic condition.
2. To better understand the environmental fates of PAEs in the environment.
3. To also use immobilized cells for PAE degradation as they have also been found to be more efficient in PAE biodegradation than free cells. This aims to investigate if there will be any difference in the results.
4. Further research to be undertaken to investigate the characteristic features (physico-chemical, physiological, biological, etc) of *E. coli*, *S. aureus* and *A. niger* responsible for the high efficiency DEP and DBP degrading abilities, as was observed in this present work. This research did not investigate these features.

APPENDICES

1) Autoaggregation kinetics of *E. coli*, *S. aureus* and *A. niger* with or without 50 µg mL⁻¹ DEP.

Time (min)	<i>E. coli</i>		<i>S. aureus</i>		<i>A. niger</i>	
	0 µg mL ⁻¹	50 µg mL ⁻¹	0 µg mL ⁻¹	50 µg mL ⁻¹	0 µg mL ⁻¹	50 µg mL ⁻¹

s)						
0	1.02 0±2. 3E ⁻³	0.734± 2.0E ⁻³	1.706± 3.2E ⁻³	1.361 ±1.3E ⁻³	0.98 5±3. 1E ⁻³	0.658±2.1 E ⁻³
10	0.99 1±2. 1E ⁻³	0.673± 2.1E ⁻³	1.685± 2.8E ⁻³	1.293 ±1.6E ⁻³	0.96 2±2. 9E ⁻³	0.592±1.5 E ⁻³
20	0.97 5±1. 9E ⁻³	0.613± 1.8E ⁻³	1.666± 2.5E ⁻³	1.221 ±1.1E ⁻³	0.93 5±2. 3E ⁻³	0.529±0.9 E ⁻³
30	0.96 3±1. 9E ⁻³	0.554± 1.6E ⁻³	1.629± 2.2E ⁻³	1.149 ±2.1E ⁻³	0.90 8±2. 5E ⁻³	0.466±1.8 E ⁻³
40	0.93 6±1. 7E ⁻³	0.495± 1.7E ⁻³	1.592± 2.3E ⁻³	1.077 ±1.2E ⁻³	0.88 1±2. 7E ⁻³	0.403±1.2 E ⁻³
50	0.90 9±1. 8E ⁻³	0.435± 1.5E ⁻³	1.555± 2.1E ⁻³	1.005 ±2.2E ⁻³	0.85 4±2. 3E ⁻³	0.340±1.4 E ⁻³
60	0.88 2±1. 5E ⁻³	0.375± 1.3E ⁻³	1.518± 2.2E ⁻³	0.933 ±1.3E ⁻³	0.82 7±2. 3E ⁻³	0.277±1.6 E ⁻³
90	0.85 5±1. 3E ⁻³	0.317± 1.2E ⁻³	1.481± 2.0E ⁻³	0.861 ±1.5E ⁻³	0.80 0±2. 1E ⁻³	0.214±1.0 E ⁻³
120	0.82 8±1. 1E ⁻³	0.257± 1.2E ⁻³	1.444± 1.9E ⁻³	0.789 ±1.2E ⁻³	0.77 3±2. 2E ⁻³	0.151±0.8 E ⁻³

2) Autoaggregation kinetics of *E. coli*, *S. aureus* and *A. niger* with or without 50 µg mL⁻¹ DBP.

Time (mins)	<i>E. coli</i>		<i>S. aureus</i>		<i>A. niger</i>	
	0 µg mL ⁻¹	50 µg mL ⁻¹	0 µg mL ⁻¹	50 µg mL ⁻¹	0 µg mL ⁻¹	50 µg mL ⁻¹

)						
0	1.020± 1.2E ⁻³	0.857± 1.0E ⁻³	1.706± 1.9E ⁻³	1.546± 1.7E ⁻³	0.985± 1.5E ⁻³	0.849± 1.8E ⁻³
10	0.991± 1.1E ⁻³	0.830± 0.9E ⁻³	1.685± 1.8E ⁻³	1.508± 1.6E ⁻³	0.962± 1.2E ⁻³	0.817± 1.6E ⁻³
20	0.975± 1.2E ⁻³	0.804± 0.8E ⁻³	1.666± 1.8E ⁻³	1.492± 1.5E ⁻³	0.935± 1.4E ⁻³	0.790± 1.4E ⁻³
30	0.963± 1.0E ⁻³	0.780± 0.9E ⁻³	1.629± 1.9E ⁻³	1.440± 1.3E ⁻³	0.908± 1.3E ⁻³	0.763± 1.3E ⁻³
40	0.936± 1.1E ⁻³	0.754± 1.0E ⁻³	1.592± 1.7E ⁻³	1.388± 1.4E ⁻³	0.881± 1.2E ⁻³	0.736± 1.1E ⁻³
50	0.909± 1.1E ⁻³	0.729± 0.8E ⁻³	1.555± 1.5E ⁻³	1.336± 1.3E ⁻³	0.854± 1.0E ⁻³	0.708± 0.9E ⁻³
60	0.882± 1.0E ⁻³	0.704± 0.6E ⁻³	1.518± 1.6E ⁻³	1.284± 1.2E ⁻³	0.827± 1.1E ⁻³	0.681± 1.0E ⁻³
90	0.855± 0.8E ⁻³	0.679± 0.7E ⁻³	1.481± 1.7E ⁻³	1.232± 1.4E ⁻³	0.800± 1.2E ⁻³	0.654± 0.8E ⁻³
12 0	0.828± 0.9E ⁻³	0.654± 0.8E ⁻³	1.444± 1.4E ⁻³	1.180± 1.3E ⁻³	0.773± 1.0E ⁻³	0.627± 0.6E ⁻³

3) % hydrophobicity and % autoaggregation abilities of strains grown in medium containing 50 µg mL⁻¹ DEP or DBP.

DEP		DBP	
% <i>SHb</i>	% <i>A_{Ag}</i>	% <i>SHb</i>	% <i>A_{Ag}</i>
64.90 ±0.5 9	63.00±0 .52	64.90±0.5 2	63.00±0.56
57.70 ±0.4 8	43.25±0 .50	43.85±0.5 1	35.00±0.52
79.55 ±0.4 7	78.25±0 .47	79.55±0.5 2	78.25±0.49
65.95 ±0.4	65.00±0 .45	59.40±0.4 9	58.00±0.45

4			
60.30 ±0.4 1	56.25±0 .43	60.30±0.4 6	56.25±0.42
43.10 ±0.4 3	36.25±0 .39	31.70±0.4 4	23.00±0.39

4) Surface hydrophobicity (%*SHb*) of the three microbial cells grown in their respective medium only or a medium containing 50 µg mL⁻¹ DEP or DBP.

Microbe	Blank	DEP	DBP
<i>E. coli</i>	64.90±0.99	43.85±1.86	57.70±0.97
<i>S. aureus</i>	79.55±2.98	59.40±2.18	65.95±1.68
<i>A. niger</i>	60.30±0.73	31.70±1.03	43.10±0.79

5) Autoaggregation ability (%*A_{Ag}*) of the three microbial cells grown in their respective medium only or a medium containing 50 µg mL⁻¹ DEP or DBP.

Microbe	Blank	DEP	DBP
<i>E. coli</i>	63.00±2.09	43.25±0.71	35.00±1.23
<i>S. aureus</i>	78.25±2.86	65.00±2.13	58.00±2.65
<i>A. niger</i>	56.25±2.11	36.25±1.02	23.00±0.45

6) Mean values of microbial cell surface hydrophobicity (CSH), DEP degradation efficiency and inhibition (*IC*₅₀).

Microbes	<i>IC</i> ₅₀ (µg mL ⁻¹)	CSH (%)	% mean DEP Degradation
----------	--	---------	------------------------

<i>E. coli</i>	197.37±2.26	64.90±0.58	83.83±0.91
<i>S. aureus</i>	154.43±1.82	78.25±0.62	96.18±0.93
<i>A. niger</i>	127.84±1.35	51.30±0.43	83.97±0.92

7) Mean values of microbial cell surface hydrophobicity (CSH), DBP degradation efficiency and inhibition (IC_{50}).

M ic ro be s	IC_{50} ($\mu\text{g mL}^{-1}$)	CSH (%)	% mean DBP Degradatio n
<i>E. co li</i>	154.43±2.0 2	64.90±0.7 5	85.10±0.84
<i>S. au re us</i>	137.95±1.9 8	78.25±0.8 1	75.95±0.79
<i>A. ni ge r</i>	155.68±2.3 8	51.30±0.4 3	97.27±0.94

8) Biomass ($\mu\text{g mL}^{-1}$) of 200 $\mu\text{g mL}^{-1}$ PAE culture medium

a) 200 $\mu\text{g mL}^{-1}$ DEP culture medium

200 $\mu\text{g mL}^{-1}$ DEP					
Time , t (h)	Control 1	Control 2	<i>E. coli</i>	<i>S. aureus</i>	<i>A. niger</i>
0	0.00	0.00	0.00	0.00	0.00
4	0.40±0.2 E ⁻³	0.20±0.1 E ⁻³	0.10±0.1 E ⁻³	0.10±0.1 E ⁻³	0.20±0.1 E ⁻³
8	0.80±0.3 E ⁻³	0.50±0.3 E ⁻³	0.15±0.1 E ⁻³	0.25±0.1 E ⁻³	0.35±0.2 E ⁻³
12	1.30±0.6 E ⁻³	1.10±0.5 E ⁻³	0.40±0.2 E ⁻³	0.50±0.2 E ⁻³	0.65±0.3 E ⁻³
16	2.10±0.7	1.50±0.6	0.70±0.3	0.90±0.3	1.00±0.5

	E ⁻³	E ⁻³	E ⁻³	E ⁻³	E ⁻³
20	3.10±0.7 E ⁻³	2.40±0.7 E ⁻³	1.30±0.5 E ⁻³	1.70±0.6 E ⁻³	2.10±0.7 E ⁻³
40	3.50±0.8 E ⁻³	3.00±0.9 E ⁻³	1.90±0.7 E ⁻³	2.30±0.7 E ⁻³	2.60±0.7 E ⁻³
60	3.70±0.8 E ⁻³	3.20±0.9 E ⁻³	2.20±0.7 E ⁻³	2.50±0.7 E ⁻³	2.80±0.7 E ⁻³
80	3.00±0.7 E ⁻³	2.80±0.8 E ⁻³	2.00±0.7 E ⁻³	2.30±0.7 E ⁻³	2.50±0.7 E ⁻³
100	2.60±0.5 E ⁻³	2.30±0.7 E ⁻³	1.70±0.6 E ⁻³	1.90±0.5 E ⁻³	2.10±0.6 E ⁻³

b) 200 µg mL⁻¹DBP culture medium

200 µg mL ⁻¹ DBP					
Time , <i>t</i> (h)	Control 1	Control 2	<i>E. coli</i>	<i>S. aureus</i>	<i>A. niger</i>
0	0.00	0.00	0.00	0.00	0.00
5	0.40±0.2 E ⁻³	0.20±0.1 E ⁻³	0.10±0.1 E ⁻³	0.10±0.1 E ⁻³	0.20±0.1 E ⁻³
10	0.80±0.3 E ⁻³	0.50±0.3 E ⁻³	0.25±0.1 E ⁻³	0.15±0.1 E ⁻³	0.35±0.2 E ⁻³
15	1.30±0.6 E ⁻³	1.10±0.5 E ⁻³	0.50±0.3 E ⁻³	0.40±0.2 E ⁻³	0.65±0.3 E ⁻³
20	2.10±0.7 E ⁻³	1.50±0.6 E ⁻³	0.90±0.4 E ⁻³	0.70±0.3 E ⁻³	1.00±0.4 E ⁻³
40	3.10±0.7 E ⁻³	2.40±0.7 E ⁻³	1.40±0.6 E ⁻³	0.90±0.4 E ⁻³	1.70±0.7 E ⁻³
60	3.50±0.8 E ⁻³	3.00±0.9 E ⁻³	1.90±0.7 E ⁻³	1.50±0.6 E ⁻³	2.20±0.7 E ⁻³
80	3.70±0.8 E ⁻³	3.20±0.9 E ⁻³	2.10±0.7 E ⁻³	1.80±0.7 E ⁻³	2.40±0.7 E ⁻³
100	3.00±0.7 E ⁻³	2.80±0.8 E ⁻³	1.90±0.7 E ⁻³	1.60±0.7 E ⁻³	2.10±0.7 E ⁻³
120	2.60±0.5 E ⁻³	2.30±0.7 E ⁻³	1.50±0.5 E ⁻³	1.30±0.6 E ⁻³	1.70±0.6 E ⁻³

9) Change in degradation efficiency, C/C_0 , with Time.

a) *E. coli* + DEP

<i>E. coli</i> + DEP				
Time, <i>t</i> (h)	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
		1	1	1
0	1.000±1.3E ⁻³	1.000±1.3E ⁻³	1.000±1.3E ⁻³	1.000±1.3E ⁻³
6	0.087±1.3E ⁻³	0.380±1.3E ⁻³	0.515±1.3E ⁻³	0.688±1.3E ⁻³
12	0.000	0.103±1.3E ⁻³	0.228±1.3E ⁻³	0.383±1.3E ⁻³
18	-	0.042±1.3E ⁻³	0.132±1.3E ⁻³	0.214±1.3E ⁻³
24	-	0.000	0.067±1.3E ⁻³	0.166±1.3E ⁻³
30	-	-	0.033±1.3E ⁻³	0.119±1.3E ⁻³
36	-	-	0.000	0.085±1.3E ⁻³
42	-	-	-	0.052±1.3E ⁻³
48	-	-	-	0.027±1.3E ⁻³
54	-	-	-	0.000

“-“ means “**not** detected”

b) *S. aureus* + DEP

S. aureus + DEP				
Time, t (h)	50 µg mL⁻¹	100 µg mL⁻¹	200 µg mL⁻¹	300 µg mL⁻¹
0	1.000±1.0E ⁻³	1.000±1.0E ⁻³	1.000±1.0E ⁻³	1.000±1.0E ⁻³
8	0.111±1.0E ⁻³	0.458±1.0E ⁻³	0.583±1.0E ⁻³	0.713±1.0E ⁻³
16	0.032±1.0E ⁻³	0.175±1.0E ⁻³	0.327±1.0E ⁻³	0.450±1.0E ⁻³
24	0.000	0.116±1.0E ⁻³	0.216±1.0E ⁻³	0.297±1.0E ⁻³
32	-	0.072±1.0E ⁻³	0.146±1.0E ⁻³	0.236±1.0E ⁻³
40	-	0.031±1.0E ⁻³	0.105±1.0E ⁻³	0.174±1.0E ⁻³
48	-	0.000	0.062±1.0E ⁻³	0.139±1.0E ⁻³
56	-	-	0.029±1.0E ⁻³	0.092±1.0E ⁻³
64	-	-	0.000	0.048±1.0E ⁻³
72	-	-	-	0.000

“-“ means “**not** detected”

c) *A. niger* + DEP

<i>A. niger</i> + DEP				
Time, t (h)	50 µg mL⁻¹	100 µg mL⁻¹	200 µg mL⁻¹	300 µg mL⁻¹
0	1.000±1.0E ⁻³	1.000±1.0E ⁻³	1.000±1.0E ⁻³	1.000±1.0E ⁻³
3	0.180±1.0E ⁻³	0.575±1.0E ⁻³	0.683±1.0E ⁻³	0.813±1.0E ⁻³
6	0.000	0.265±1.0E ⁻³	0.483±1.0E ⁻³	0.650±1.0E ⁻³
12	-	0.101±1.0E ⁻³	0.234±1.0E ⁻³	0.401±1.0E ⁻³
24	-	0.068±1.0E ⁻³	0.154±1.0E ⁻³	0.271±1.0E ⁻³
36	-	0.027±1.0E ⁻³	0.113±1.0E ⁻³	0.200±1.0E ⁻³
48	-	0.000	0.069±1.0E ⁻³	0.145±1.0E ⁻³
60	-	-	0.035±1.0E ⁻³	0.092±1.0E ⁻³

72	-	-	0.000	0.045±1.0E ⁻³
84	-	-	-	0.000

“-“ means “**not** detected”

d) *E. coli* + *DBP*

<i>E. coli</i> + <i>DBP</i>				
Time, <i>t</i> (h)	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	1.000 ±1.0E -3	1.000 ±1.0E -3	1.000 ±1.0E -3	1.000±1 .0E ⁻³
3	0.310 ±1.0E -3	0.580 ±1.0E -3	0.700 ±1.0E -3	0.850±1 .0E ⁻³
6	0.090 ±1.0E -3	0.310 ±1.0E -3	0.555 ±1.0E -3	0.690±1 .0E ⁻³
12	0.000	0.080 ±1.0E -3	0.244 ±1.0E -3	0.480±1 .0E ⁻³
24	-	0.000	0.106 ±1.0E -3	0.210±1 .0E ⁻³
30	-	-	0.056 ±1.0E -3	0.150±1 .0E ⁻³
36	-	-	0.000	0.098±1 .0E ⁻³
42	-	-	-	0.064±1 .0E ⁻³
48	-	-	-	0.039±1 .0E ⁻³
54	-	-	-	0.000

“-“ means “**not** detected”

e) *S. aureus*+ DBP

<i>S. aureus</i> + DBP				
Time, <i>t</i> (h)	50 µg mL ⁻¹	100 µg mL ⁻¹ 1	200 µg mL ⁻¹ 1	300 µg mL ⁻¹ 1
0	1.000±1.0E ⁻³	1.000±1.0E ⁻³	1.000±1.0E ⁻³	1.000±1.0E ⁻³
6	0.300±1.0E ⁻³	0.600±1.0E ⁻³	0.700±1.0E ⁻³	0.813±1.0E ⁻³
12	0.120±1.0E ⁻³	0.250±1.0E ⁻³	0.354±1.0E ⁻³	0.600±1.0E ⁻³
24	0.000	0.120±1.0E ⁻³	0.218±1.0E ⁻³	0.308±1.0E ⁻³
48	-	0.062±1.0E ⁻³	0.127±1.0E ⁻³	0.208±1.0E ⁻³
60	-	0.036±1.0E ⁻³	0.101±1.0E ⁻³	0.161±1.0E ⁻³
72	-	0.000	0.068±1.0E ⁻³	0.130±1.0E ⁻³
84	-	-	0.043±1.0E ⁻³	0.096±1.0E ⁻³
96	-	-	0.000	0.041±1.0E ⁻³
108	-	-	-	0.000

“-“ means “**not** detected”

f) *A. niger* + DBP

<i>A. niger</i> + DBP				
Ti me, <i>t</i> (h)	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	1.00 0±1. 0E ⁻³	1.000 ±1.0E ⁻³	1.000± 1.0E ⁻³	1.000 ±1.0E ⁻³
9	0.20 0±1.	0.510 ±1.0E	0.650± 1.0E ⁻³	0.800 ±1.0E

	0E ⁻³	-3		-3
18	0.08 0±1. 0E ⁻³	0.200 ±1.0E -3	0.400± 1.0E ⁻³	0.590 ±1.0E -3
27	0.00 0	0.080 ±1.0E -3	0.250± 1.0E ⁻³	0.420 ±1.0E -3
36	-	0.000	0.180± 1.0E ⁻³	0.330 ±1.0E -3
45	-	-	0.124± 1.0E ⁻³	0.256 ±1.0E -3
54	-	-	0.074± 1.0E ⁻³	0.199 ±1.0E -3
63	-	-	0.039± 1.0E ⁻³	0.137 ±1.0E -3
72	-	-	0.000	0.070 ±1.0E -3
81	-	-	-	0.000

“-“ means “**not** detected”

10) Dose/concentration versus k (x 10⁻³ min⁻¹)

Conc. (µg mL ⁻¹)	DEP			DBP		
	<i>E. coli</i>	<i>S. aureus</i>	<i>A. niger</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>A. niger</i>
	<i>k</i>	<i>k</i>	<i>K</i>	<i>k</i>	<i>K</i>	<i>k</i>
0	1.62±1. .0E ⁻³	2.61±1. .0E ⁻³	4.4 2± 1.0	3.1 2±1 .0E ⁻³	2.88±1. 0E ⁻³	2.75± 1.0E ⁻³

			E ⁻³	3		
50	1.51±1 .0E ⁻³	2.07±1 .0E ⁻³	3.3 5± 1.0 E ⁻³	2.7 5±1 .0E ⁻³	1.48±1. 0E ⁻³	2.72± 1.0E ⁻³
10 0	1.26±1 .0E ⁻³	1.68±1 .0E ⁻³	1.3 3± 1.0 E ⁻³	0.7 4±1 .0E ⁻³	1.15±1. 0E ⁻³	2.70± 1.0E ⁻³
20 0	1.38±1 .0E ⁻³	1.15±1 .0E ⁻³	0.5 6± 1.0 E ⁻³	1.8 1±1 .0E ⁻³	0.44±1. 0E ⁻³	0.57± 1.0E ⁻³
30 0	1.22±1 .0E ⁻³	1.51±1 .0E ⁻³	-	0.9 4±1 .0E ⁻³	0.37±1. 0E ⁻³	-

“-“ means “**not** detected”

11) Turbidity/Optical Density at 660 nm (OD₆₆₀)

a) *E. coli* + DEP

<i>E. coli</i> + DEP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.00	0.00	0.00	0.00	0.00
6	0.12±1.0E ⁻³	0.10±1.0E ⁻³	0.09±1.0E ⁻³	0.06±1.0E ⁻³	0.04±0.1E ⁻³
12	0.42±1.0E ⁻³	0.35±1.0E ⁻³	0.31±1.0E ⁻³	0.20±1.0E ⁻³	0.12±0.1E ⁻³
18	0.90±1.0E ⁻³	0.75±1.0E ⁻³	0.66±1.0E ⁻³	0.42±1.0E ⁻³	0.29±0.2E ⁻³
24	1.35±1.0E ⁻³	1.13±1.0E ⁻³	0.99±1.0E ⁻³	0.55±1.0E ⁻³	0.32±0.2E ⁻³
30	1.80±1.0E ⁻³	1.50±1.0E ⁻³	1.32±1.0E ⁻³	0.84±1.0E ⁻³	0.50±0.3E ⁻³
36	2.31±1.0E ⁻³	1.88±1.0E ⁻³	1.65±1.0E ⁻³	1.05±1.0E ⁻³	0.42±0.3E ⁻³
42	2.43±1.0E ⁻³	2.22±1.0E ⁻³	1.90±1.0E ⁻³	1.19±1.0E ⁻³	0.36±0.2E ⁻³
48	2.41±1.0E ⁻³	2.21±1.0E ⁻³	2.04±1.0E ⁻³	1.09±1.0E ⁻³	0.34±0.2E ⁻³
54	2.40±1.0E ⁻³	2.01±1.0E ⁻³	1.80±1.0E ⁻³	1.02±1.0E ⁻³	0.30±0.2E ⁻³

b) *S. aureus* + DEP

<i>S. aureus</i> + DEP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.000	0.000	0.000	0.000	0.000
8	0.100±1.0E ⁻³	0.090±1.0E ⁻³	0.080±1.0E ⁻³	0.060±1.0E ⁻³	0.035±0.1E ⁻³
16	0.350±1.0E ⁻³	0.310±1.0E ⁻³	0.280±1.0E ⁻³	0.190±1.0E ⁻³	0.100±0.1E ⁻³
24	0.750±1.0E ⁻³	0.650±1.0E ⁻³	0.600±1.0E ⁻³	0.430±1.0E ⁻³	0.180±0.1E ⁻³
32	1.500±1.0E ⁻³	1.000±1.0E ⁻³	1.200±1.0E ⁻³	0.800±1.0E ⁻³	0.330±0.1E ⁻³
40	1.880±1.0E ⁻³	1.700±1.0E ⁻³	1.500±1.0E ⁻³	1.050±1.0E ⁻³	0.280±0.1E ⁻³

48	2.130±1.0E ⁻³	1.900±1.0E ⁻³	1.700±1.0E ⁻³	1.200±1.0E ⁻³	0.139±0.1E ⁻³
56	2.310±1.0E ⁻³	2.150±1.0E ⁻³	1.850±1.0E ⁻³	1.350±1.0E ⁻³	0.092±0.1E ⁻³
64	2.380±1.0E ⁻³	2.000±1.0E ⁻³	1.900±1.0E ⁻³	1.250±1.0E ⁻³	0.048±0.1E ⁻³
72	2.310±1.0E ⁻³	1.900±1.0E ⁻³	1.800±1.0E ⁻³	1.220±1.0E ⁻³	0.001±0.1E ⁻³

c) *A. niger* + DEP

<i>A. niger</i> + DEP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.00	0.00	0.00	0.00	0.00
3	0.05±0.1E ⁻³	0.03±0.1E ⁻³	0.02±0.1E ⁻³	0.02±0.1E ⁻³	0.01±0.1E ⁻³
6	0.10±0.2E ⁻³	0.08±0.2E ⁻³	0.06±0.1E ⁻³	0.06±0.1E ⁻³	0.03±0.1E ⁻³
12	0.25±0.9E ⁻³	0.19±0.2E ⁻³	0.21±0.2E ⁻³	0.12±0.1E ⁻³	0.05±0.1E ⁻³
24	0.62±1.0E ⁻³	0.46±0.3E ⁻³	0.33±0.2E ⁻³	0.28±0.1E ⁻³	0.07±0.1E ⁻³
36	1.35±1.0E ⁻³	1.12±1.0E ⁻³	0.46±0.3E ⁻³	0.36±0.2E ⁻³	0.09±0.1E ⁻³
48	1.75±1.1E ⁻³	1.47±1.0E ⁻³	0.69±0.4E ⁻³	0.43±0.3E ⁻³	0.11±0.1E ⁻³
60	1.98±1.2E ⁻³	1.61±1.1E ⁻³	0.77±0.6E ⁻³	0.34±0.4E ⁻³	0.15±0.2E ⁻³
72	1.96±1.1E ⁻³	1.55±1.2E ⁻³	0.65±0.4E ⁻³	0.29±0.3E ⁻³	0.12±0.2E ⁻³
84	1.92±1.3E ⁻³	1.32±1.2E ⁻³	0.55±0.4E ⁻³	0.27±0.3E ⁻³	0.07±0.1E ⁻³

d) *E. coli* + DBP

<i>E. coli</i> + DBP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.00	0.00	0.00	0.00	0.00
3	0.13±1.1E ⁻³	0.10±1.0E ⁻³	0.07±0.8E ⁻³	0.05±0.2E ⁻³	0.03±0.2E ⁻³

6	0.22±1.2E ⁻ 3	0.16±1.1E ⁻ 3	0.12±0.9E ⁻ 3	0.09±0.3E ⁻ 3	0.06±0.2E ⁻ 3
12	0.46±1.1E ⁻ 3	0.39±1.2E ⁻ 3	0.26±0.8E ⁻ 3	0.15±0.3E ⁻ 3	0.10±0.3E ⁻ 3
24	1.44±1.3E ⁻ 3	1.28±1.2E ⁻ 3	1.11±0.9E ⁻ 3	0.71±0.5E ⁻ 3	0.52±0.4E ⁻ 3
30	1.92±1.2E ⁻ 3	1.65±1.3E ⁻ 3	1.33±0.9E ⁻ 3	1.06±0.8E ⁻ 3	0.73±0.4E ⁻ 3
36	2.40±1.4E ⁻ 3	1.98±1.3E ⁻ 3	1.57±1.0E ⁻ 3	1.28±1.0E ⁻ 3	0.99±0.4E ⁻ 3
42	2.72±1.4E ⁻ 3	2.13±1.4E ⁻ 3	1.74±1.1E ⁻ 3	1.35±1.0E ⁻ 3	1.07±0.5E ⁻ 3
48	2.96±1.3E ⁻ 3	2.07±1.4E ⁻ 3	1.69±1.2E ⁻ 3	1.29±1.1E ⁻ 3	0.88±0.5E ⁻ 3
54	3.04±1.4E ⁻ 3	2.01±1.4E ⁻ 3	1.45±1.2E ⁻ 3	1.17±1.1E ⁻ 3	0.74±0.6E ⁻ 3

e) *S. aureus* + DBP

<i>S. aureus</i> + DBP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹ 1	200 µg mL ⁻¹ 1	300 µg mL ⁻¹ 1
0	0.000	0.000	0.000	0.000	0.000
6	0.040±1.0E ⁻ 3	0.030±1.0E ⁻ 3	0.020±1.0E ⁻ 3	0.010±1.0E ⁻ 3	0.010±0.1E ⁻ 3
12	0.090±1.0E ⁻ 3	0.060±1.0E ⁻ 3	0.045±1.0E ⁻ 3	0.028±1.0E ⁻ 3	0.010±0.1E ⁻ 3
24	0.370±1.0E ⁻ 3	0.160±1.0E ⁻ 3	0.120±1.0E ⁻ 3	0.060±1.0E ⁻ 3	0.030±0.1E ⁻ 3
48	0.990±1.0E ⁻ 3	0.560±1.0E ⁻ 3	0.340±1.0E ⁻ 3	0.110±1.0E ⁻ 3	0.080±0.1E ⁻ 3
60	1.320±1.0E ⁻ 3	0.750±1.0E ⁻ 3	0.570±1.0E ⁻ 3	0.290±1.0E ⁻ 3	0.110±0.1E ⁻ 3
72	1.650±1.0E ⁻ 3	1.010±1.0E ⁻ 3	0.730±1.0E ⁻ 3	0.490±1.0E ⁻ 3	0.210±0.1E ⁻ 3
84	1.870±1.0E ⁻ 3	1.150±1.0E ⁻ 3	0.910±1.0E ⁻ 3	0.550±1.0E ⁻ 3	0.130±0.1E ⁻ 3
96	2.040±1.0E ⁻ 3	1.030±1.0E ⁻ 3	0.850±1.0E ⁻ 3	0.460±1.0E ⁻ 3	0.110±0.1E ⁻ 3
108	2.090±1.0E ⁻ 3	0.990±1.0E ⁻ 3	0.710±1.0E ⁻ 3	0.340±1.0E ⁻ 3	0.070±0.1E ⁻ 3

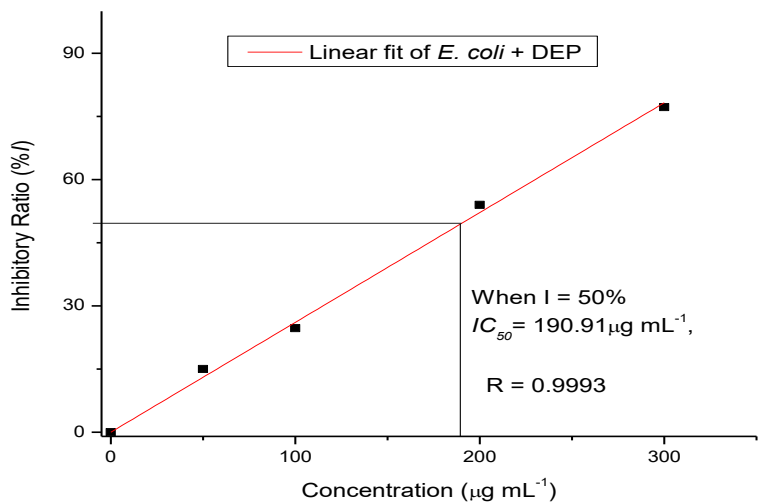
f) *A. niger* + DBP

<i>A. niger</i> + DBP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
			1	1	1
0	0.000	0.000	0.000	0.000	0.000
9	0.104±1.0E ⁻³	0.080±1.0E ⁻³	0.050±1.0E ⁻³	0.025±1.0E ⁻³	0.010±1.0E ⁻³
18	0.364±1.0E ⁻³	0.280±1.0E ⁻³	0.160±1.0E ⁻³	0.090±1.0E ⁻³	0.040±1.0E ⁻³
27	0.780±1.0E ⁻³	0.600±1.0E ⁻³	0.340±1.0E ⁻³	0.120±1.0E ⁻³	0.070±1.0E ⁻³
36	1.170±1.0E ⁻³	0.900±1.0E ⁻³	0.580±1.0E ⁻³	0.330±1.0E ⁻³	0.090±1.0E ⁻³
45	1.560±1.0E ⁻³	1.200±1.0E ⁻³	0.970±1.0E ⁻³	0.610±1.0E ⁻³	0.100±1.0E ⁻³
54	1.950±1.0E ⁻³	1.500±1.0E ⁻³	1.190±1.0E ⁻³	0.740±1.0E ⁻³	0.170±1.0E ⁻³
63	2.210±1.0E ⁻³	1.700±1.0E ⁻³	1.230±1.0E ⁻³	0.770±1.0E ⁻³	0.150±1.0E ⁻³
72	2.405±1.0E ⁻³	1.620±1.0E ⁻³	1.110±1.0E ⁻³	0.680±1.0E ⁻³	0.120±1.0E ⁻³
81	2.470±1.0E ⁻³	1.590±1.0E ⁻³	0.890±1.0E ⁻³	0.650±1.0E ⁻³	0.080±1.0E ⁻³

12) Calculated % Inhibition values from turbidity/*OD*₆₆₀ values (Appendix 11).

a) (i) Mean values of Inhibitory Ratios for *E. coli* + DEP from *OD*₆₆₀ values.

<i>E. coli</i> + DEP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.00	0.00	0.00	0.00	0.00
6	0.00	16.70	25.00	50.00	66.70
12	0.00	16.70	26.20	52.40	71.40
18	0.00	16.70	26.70	53.30	67.80
24	0.00	16.30	26.70	59.30	76.30
30	0.00	16.70	26.70	53.30	72.20
36	0.00	18.60	28.60	54.50	81.80
42	0.00	8.64	21.80	51.00	85.20
48	0.00	8.30	15.40	54.80	85.90
54	0.00	16.30	25.00	57.50	87.50
Mean	0.00%	14.99%	24.68%	54.01%	77.20%

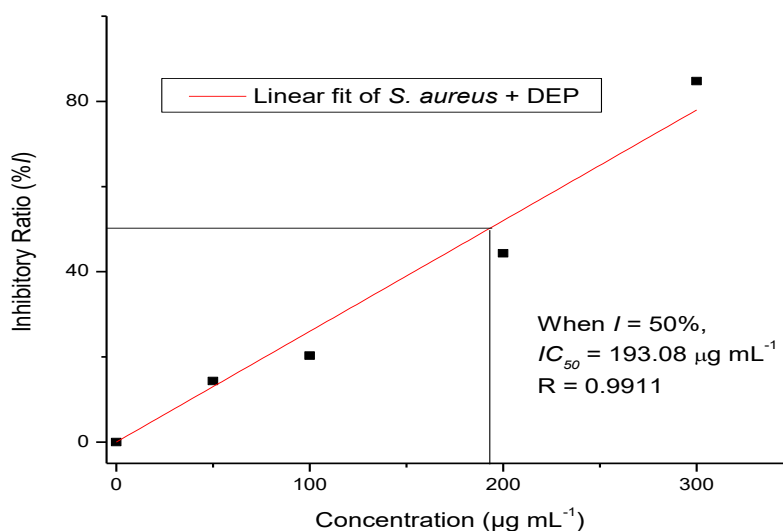


a) (ii) Calculating *IC*₅₀ for *E. coli* + DEP from concentration versus %*I* plot.

b) (i) Mean values of Inhibitory Ratios for *S. aureus* + DEP from *OD*₆₆₀ values.

<i>S. aureus</i> + DEP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.00	0.00	0.00	0.00	0.00
8	0.00	10.00	20.00	40.00	65.00
16	0.00	11.43	20.00	45.71	71.42
24	0.00	13.33	20.00	42.67	76.00

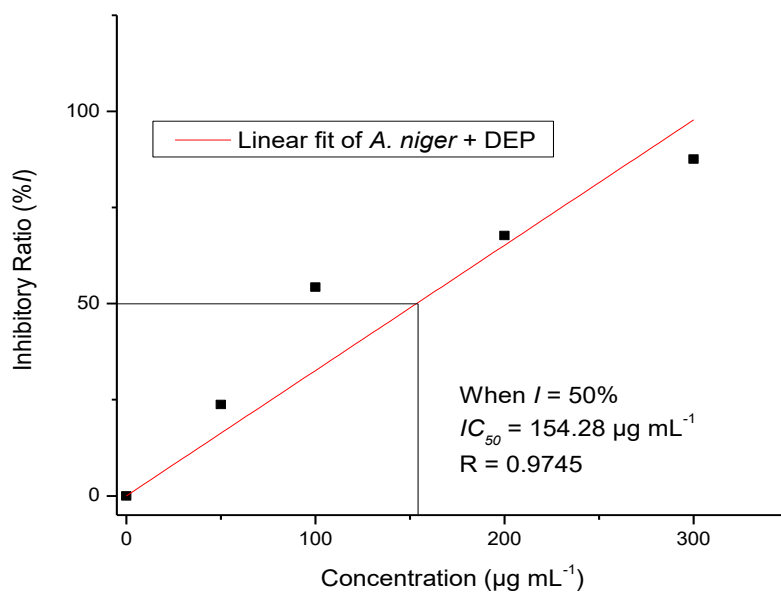
32	0.00	33.33	20.00	46.67	78.00
40	0.00	9.57	20.21	44.15	85.11
48	0.00	10.80	20.19	43.66	93.47
56	0.00	6.93	19.91	41.56	96.02
64	0.00	15.97	20.17	47.48	97.98
72	0.00	17.75	22.08	47.19	99.96
Mean	0.00%	14.35%	20.28%	44.34%	84.77%



b) (ii) Calculating IC_{50} for *S. aureus* + DEP from concentration versus %I plot.

c) (i) Mean values of Inhibitory Ratios for *A. niger* + DEP from OD_{660} values.

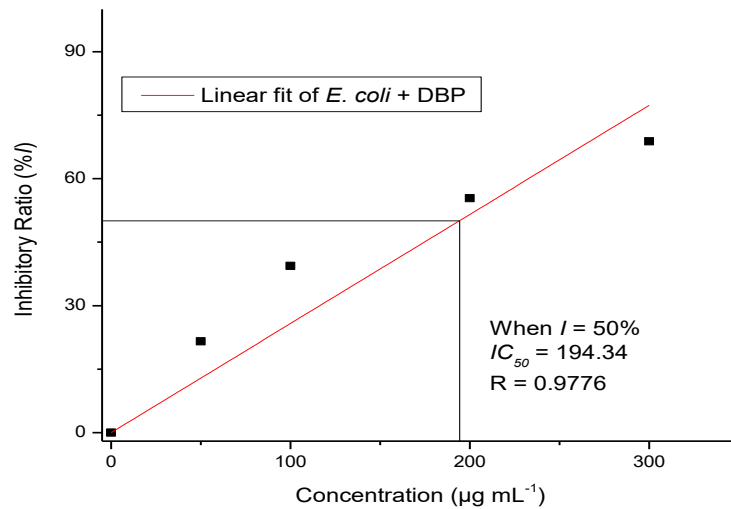
<i>A. niger</i> + DEP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.00	0.00	0.00	0.00	0.00
3	0.00	40.00	60.00	60.00	80.00
6	0.00	20.00	40.00	40.00	70.00
12	0.00	24.00	16.00	52.00	80.00
24	0.00	25.81	46.77	54.84	88.71
36	0.00	17.04	65.93	73.33	93.33
48	0.00	16.00	60.57	75.43	93.71
60	0.00	18.69	61.11	82.83	92.42
72	0.00	20.92	66.84	85.20	93.88
84	0.00	31.25	71.35	85.94	96.35
Mean	0.00%	23.75%	54.29%	67.73%	87.60%



c) (ii) Calculating IC_{50} for *A. niger* + DEP from concentration versus %*I* plot.

d) (i) Mean values of Inhibitory Ratios for *E. coli* + DBP from OD_{660} values.

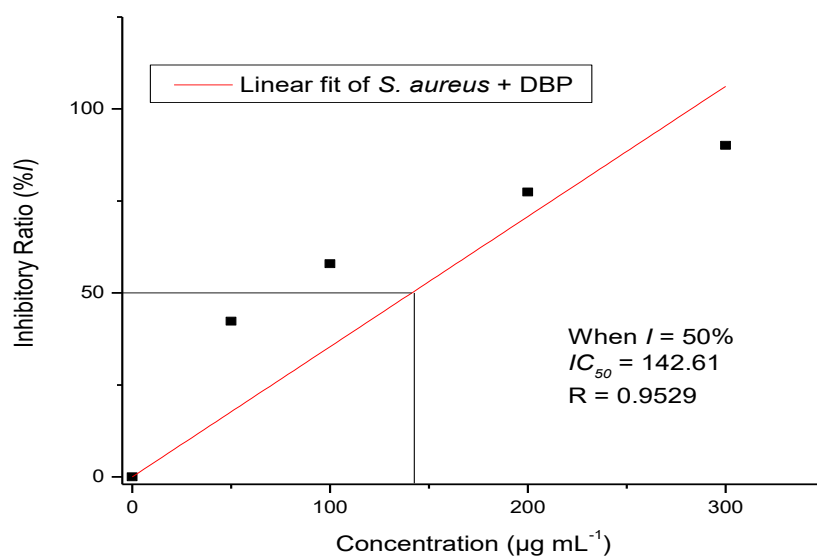
<i>E. coli</i> + DBP					
Time, <i>t</i> (h)	0 $\mu\text{g mL}^{-1}$	50 $\mu\text{g mL}^{-1}$	100 $\mu\text{g mL}^{-1}$	200 $\mu\text{g mL}^{-1}$	300 $\mu\text{g mL}^{-1}$
0	0.00	0.00	0.00	0.00	0.00
3	0.00	23.08	46.15	61.54	76.92
6	0.00	27.27	45.45	59.09	72.73
12	0.00	15.22	43.48	67.39	78.26
24	0.00	11.11	22.92	50.69	63.89
30	0.00	14.06	30.73	44.79	61.98
36	0.00	17.50	34.58	46.67	58.75
42	0.00	21.69	36.03	50.37	60.66
48	0.00	30.07	42.91	56.42	70.27
54	0.00	33.88	52.30	61.51	75.66
Mean	0.00%	21.54%	39.39%	55.39%	68.79%



d) (ii) Calculating IC_{50} for *E. coli* + DBP from concentration versus %*I* plot.

e) (i) Mean values of Inhibitory Ratios for *S. aureus* + DBP from OD_{660} values.

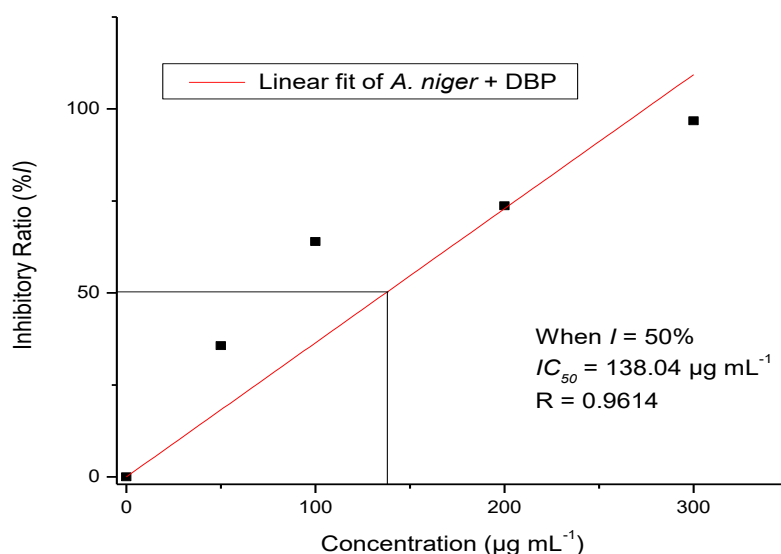
<i>S. aureus</i> + DBP					
Time, <i>t</i> (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.00	0.00	0.00	0.00	0.00
6	0.00	25.00	50.00	75.00	75.00
12	0.00	33.33	50.00	68.89	88.89
24	0.00	56.76	67.57	83.78	91.89
48	0.00	43.43	65.66	88.89	91.92
60	0.00	43.18	56.82	78.03	91.67
72	0.00	38.79	55.76	70.30	87.27
84	0.00	38.50	51.34	70.59	93.05
96	0.00	49.51	58.33	77.45	94.61
108	0.00	52.63	66.03	83.73	96.65
Mean	0.00%	42.35%	57.95%	77.41%	90.11%



e) (ii) Calculating IC_{50} for *S. aureus* + DBP from concentration versus % I plot.

f) (i) Mean values of Inhibitory Ratios for *A. niger* + DBP from OD_{660} values.

<i>A. niger</i> + DBP					
Time, t (h)	0 µg mL ⁻¹	50 µg mL ⁻¹	100 µg mL ⁻¹	200 µg mL ⁻¹	300 µg mL ⁻¹
0	0.00	0.00	0.00	0.00	0.00
9	0.00	23.08	51.92	75.96	90.38
18	0.00	23.08	56.04	75.27	89.01
27	0.00	23.08	56.41	84.62	91.03
36	0.00	23.08	50.43	71.79	92.31
45	0.00	23.08	37.82	60.90	93.59
54	0.00	23.08	38.97	62.05	91.28
63	0.00	23.08	44.34	65.16	93.21
72	0.00	32.64	53.85	71.73	95.01
81	0.00	35.63	63.97	73.68	96.76
Mean	0.00%	35.63%	63.97%	73.68%	96.76%



f) (ii) Calculating IC_{50} for *A. niger* + DBP from concentration versus %I plot.

REFERENCES

- Adhoum, N., Monser, L., 2004. Removal of phthalate on modified activated carbon application to the treatment of industrial wastewater, *Sep. Purif. Technol.* 38: 233–239.
- Ahn, J. Y., Kim, Y. H., Min, J., Lee, J., 2006. Accelerated degradation of dipentyl phthalate by *Fusarium oxysporum* f. sp *pisi* cutinase and toxicity evaluation of its degradation products using bioluminescent bacteria. *Curr Microbiol* 52:340–344
- Alatrisme-Mondragon, F., Iranpour, R., Ahring, B. K., 2003. Toxicity of di-(2-ethylhexyl) phthalate on the anaerobic digestion of wastewater sludge. *Water Res* 37:1260–1269
- Albro, P. W., Thomas, R. W., Fishbein, L. 1973. Metabolism of diethyl hexyl phthalate by rats: isolation and characterisation of urinary metabolism. *Journal of Chromatography* 76:321–330.
- Al-Tahhan, R.A., Sandrin, T.R., Bodour, A.A., Maier, R.M., 2000. Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: Effect on cell surface properties and interaction with hydrophobic substrates. *Appl. Environ. Microbiol.* 66:3262–3268.
- Amir, S., Hafidi, M., Merlina, G., Hamdi, H., Jouraiphy, A., El Gharous, M., Revel, J. C. 2005. Fate of phthalic acid esters during composting of both lagooning and activated sludges. *Process Biochem* 40:2183–2190
- Arikan, S., Lozano-Chiu, M., Paetznick, V., Rex, J. H., 2001. In vitro susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. *Antimicrob. Agents Chemother.* 45:327–330.
- Ayranci, E., Bayram, E., 2005. Adsorption of phthalic acid and its esters onto high-area activated carbon-cloth studied by *in situ* UV-spectroscopy. *Journal of hazardous materials* 122(1-2):147–153.
- Bago, B., Martin, Y., Mejia, G., Broto-Puig, F., Diaz-Ferrero, J., Agut, M., Comellas, L., 2005. Di-(2-ethylhexyl) phthalate in sewage sludge and post-treated sludge: quantitative determination by HRGC-MS and mass spectral characterization. *Chemosphere* 59:1191–1195
- Balafas, D., Shaw, K. J., Whitfield, F. B., 1999. Phthalate and adipate esters in Australian packaging materials. *Food Chem.*, 65:279–288.
- Barrett, J. R., 2005. Phthalates and Baby Boys: Potential Disruption of Human Genital Development. *Environ Health Perspect.* 113(8): A542.
- Barros, N., Feijó, S., Simoni, J. A., Prado, A. G. S., Barboza, F. D., Airolidi, C., 1999. Microcalorimetric study of some Amazonian soils. *Thermochim. Acta* 328:99.
- Battersby, N. S., Wilson, V., 1989. Survey of the anaerobic biodegradation potential of organic-chemicals in digesting sludge. *Appl Environ Microbiol* 55:433–439
- Benckiser G, Ottow JCG (1982) Metabolism of the plasticizer di-normal-butylphthalate by *Pseudomonas pseudoalcaligenes* under anaerobic conditions, with nitrate as the only electron-acceptor. *Appl Environ Microbiol* 44:576–578

15. Bodzek, M., Dudziak, M., Luks-Betlej, K., 2004. Application of membrane techniques to water purification. Removal of phthalates. *Desalination* 162:121-128.
16. Boethling, R. S., 1984. Biodegradation testing of insoluble chemicals. *Environmental Toxicology and Chemistry* 3:5-7.
17. Bornehag, C. G., Sundell, J., Weschler, C. J., Sigsgaard, T., Lundgren, B., Hasselgren, M., Hägerhed-Engman, L., 2004. The association between asthma and allergic symptoms in children and phthalates in house dust: a nested case-control study. *Environ. Health Perspect.* 112 (14): 1393-7.
18. Bouchez, M., Blanchet, D., Vandecasteele, J. P., 1995. Substrate availability in phenanthrene biodegradation: transfer mechanisms and influence on metabolism. *Appl. Microbiol. Biotechnol* 43:952-960.
19. Breslow, R., 1991. In Comprehensive Organic Synthesis, Volume 7 (Eds.: B. M. Trost, I. Fleming), Pergamon Press, Oxford, p. 39.
20. Brossa, L., Marcé, R. M., Borrull, F., Pocurull, E., 2003. Determination of endocrine-disrupting compounds in water samples by on-line solid-phase extraction-programmed-temperature vaporisation-gas chromatography-mass spectrometry. *Journal of Chromatography A* 998:41-50.
21. Brotons J. A., Olea-Serrano, M. F., Villalobos, M., Pedraza, V., Olea, N., 1995. *Environmental Health Perspectives* 103(6):608.
22. Busscher, H.J., van de Belt-Gritter, B., van der Mei, H.C., 1995. Implications of microbial adhesion to hydrocarbons for evaluating cell surface hydrophobicity 1. Zeta potentials of hydrocarbon droplets. *Colloid Surf B-Biointerf* 5:111-116.
23. Caccavo, F.Jr, Schamberger, P.C., Keiding, K., Neilson, P.H., 1997. Role of hydrophobicity in adhesion of the dissimilatory Fe (III) oxide. *Appl. Environ. Microbiol.* 63(10):3837-3843.
24. Cartwright, C. D., Owen, S. A., Thompson, I. P., Burns, R. G., 2000. Biodegradation of diethyl phthalate in soil by a novel pathway. *FEMS Microbiol Lett* 186:27-34
25. Castillo, M., Barceló, D., 1999. Identification of Polar Toxicants in Industrial Wastewaters Using Toxicity-Based Fractionation with Liquid Chromatography/Mass Spectrometry. *Anal. Chem.* 71(17):3769.
26. Chang, B. V., Yang, C. M., Cheng, C. H., Yuan, S. Y., 2004. Biodegradation of phthalate esters by two bacteria strains. *Chemosphere* 55:533-538.
27. Chang, B. V., Liao, C. S., Yuan, S. Y., 2005a. Anaerobic degradation of diethyl phthalate, di-n-butyl phthalate, and di-(2-ethylhexyl) phthalate from river sediment in Taiwan. *Chemosphere* 58:1601-1607
28. Chang, B. V., Wang, T. H., Yuan, S. Y., 2007. Biodegradation of four phthalate esters in sludge. *Chemosphere* 69:1116-1123
29. Chao, W. L., Cheng, C. Y., 2007. Effect of introduced phthalate-degrading bacteria on the diversity of indigenous bacterial communities during di-(2-ethylhexyl) phthalate (DEHP) degradation in a soil microcosm. *Chemosphere* 67:482-488
30. Chao, W. L., Lin, C. M., Shiung, I.I., Kuo, Y. L., 2006. Degradation of di-butyl-phthalate by soil bacteria. *Chemosphere* 63:1377-1383.
31. Chauret, C., Inniss, W. E., Mayfield, C. I., 1996. Biotransformation of 10 °C of Di-n-butyl Phthalate in subsurface microcosms. *Ground water* 34(5):791-794.
32. Chen, J. A., Li, X., Li, J., Cao, J., Qiu, Z. Q., Zhao, Q., Xu, C., Shu, W. Q., 2007. Degradation of environmental endocrine disruptor di-2-ethylhexyl phthalate by a newly discovered bacterium, *Microbacterium* sp strain CQ0110Y. *Appl Microbiol Biotechnol* 74:676-682
33. Chen, W. W., Schoeberl, B., Jasper, P. J., Niepel, M., Nielsen, U. B., Lauffenburger, D. A., Sorger, P. K., 2008. Input-output behavior of ErbB signaling pathways as revealed by a mass action model trained against dynamic data. *Mol Syst Biol* 5: 239
34. Cheng, H. F., Chen, S. Y., Lin, J. G., 2000. Biodegradation of di-(2-ethylhexyl) phthalate in sewage sludge. *Water Sci Technol* 41:1-6
35. China Plastics Process Industry Union (CPPIU), 2004. China Plastics Industry Yearbook. *Chemical Industry Press*, Beijing.
36. Christophe, P., Christine, B., Chantal, C., et al., 1997. Cell surface characteristics of *Lactobacillus* *casci* subsp. *casci*. *Lactobacillus* *paracasci* subsp. *Paracasci*, and *Lactobacillus* *ramnosus* strains. *Appl. Environ. Microbiol.* 63:1725-1731.
37. Churchill, S. A., Harper, J. P., Churchill, P. F., 1999. Isolation and characterization of a mycobacterium species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. *Appl. Environ. Microbiol.* 65:549-552.

38. Collier, L., Balows, A., Sussman, M., 1998. Topley & Wilson's Microbiology and Microbial Infections, 9th ed, vol. 4. Arnold, London, Sydney, Auckland, New York
39. Colón, I., Caro, D., Bourdony, C. J., Rosario, O., 2000. Identification of phthalate esters in the serum of young Puerto Rican girls with premature breast development. *Environmental Health Perspectives* 108(9):895-900.
40. Consumer Product Safety Commission (CPSC), 2001. Chronic Hazard Advisory on Diisononyl Phthalate. p. 87. <http://www.cpsc.gov/library/foia/foia01/os/dinp.pdf> p. 87.
41. Cooney, C. L., Wang, D.I. C., Mateles, R. I., 1968. Measurement of heat evolution and correlation with oxygen consumption during microbial growth. *Biotechnol. Bioeng.* 11:269-281
42. Critter, S. A. M., Airoidi, C., 2001. Application of calorimetry to microbial biodegradation studies of agrochemicals in oxisols. *J. Environ. Qual.* 30:954-959.
43. Critter, S. A. M., Freitas, S. S., Airoidi, C., 2001. Calorimetry versus respirometry for the monitoring of microbial activity in a tropical soil. *Appl. Soil Ecol.* 18:217-227.
44. Critter, S. A. M., Freitas, S. S., Airoidi, C., 2002. Microbial biomass and microcalorimetric methods in tropical soils. *Thermochim. Acta* 394:145-154.
45. Davies, R. W., 1994. Heterologous gene expression and protein secretion in *Aspergillus*. *ProgInd Microbiol.* 29:527-560.
46. Dennis, D. A., Chapman, P. J., Dagley, S., 1973. Degradation of protocatechuate in *Pseudomonas testosteroni* by a pathway involving oxidation of product of *meta* fission. *J Bacteriol* 113:521-523
47. Desvergne, B., Feige, J., Casals-Casas, C., 2009. PPAR-mediated activity of phthalates: A link to the obesity epidemic? *Mol Cell Endocrinol.* 304 (1-2): 43
48. Di Gennaro, P., Collina, E., Franzetti, A., Lasagni, M., Luridiana, A., Pitea, D., Bestetti, G., 2005. Bioremediation of diethylhexyl phthalate contaminated soil: a feasibility study in slurry- and solid-phase reactors. *Environ Sci Technol* 39:325-330
49. Doyle, R. J., 2000. Contribution of the hydrophobic effect to microbial infection, *Microbes and infection/Institut Pasteur* 2:391-400.
50. Duncan-Hewitt, W. C., 1990. Predicting the relative rate of wear of pharmaceutical compacts using the mechanical properties of their constituent crystals. *Powder Technol.* 60:265-272.
51. Eaton, R. W., 2001. Plasmid-encoded phthalate catabolic pathway in *Arthrobacter keyseri* 12B. *J Bacteriol* 183:3689-3703
52. Eaton, R. W., Ribbons, D. W., 1982. Metabolism of dibutylphthalate and phthalate by *Micrococcus* sp strain 12b. *J Bacteriol* 151:48-57
53. Edsall, J. T., 1992. Memories of early days in protein science, 1926-1940. *Protein Sci.* 1:1526-1530.
54. Efroymson, R. A., Alexander, M., 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. *Environ. Sci. Technol* 29:515-521.
55. Ejlerstson, J., Svensson, B. H., 1997. Degradation of bis (2-ethylhexyl) phthalate constituents under methanogenic conditions. *Biodegradation* 7:501-506
56. Ejlerstson, J., Houwen, F. P., Svensson, B. H., 1996a. Anaerobic degradation of diethyl phthalate and phthalic acid during incubation of municipal solid waste from a biogas digester. *Swed J Agri Res* 26:53-59
57. Ejlerstson, J., Meyerson, U., Svensson, B. H., 1996b. Anaerobic degradation of phthalic acid esters during digestion of municipal solid waste under landfilling conditions. *Biodegradation* 7:345-352
58. Ejlerstson, J., Alnervik, M., Jonsson, S., Svensson, B. H., 1997. Influence of water solubility, side-chain degradability, and side-chain structure on the degradation of phthalic acid esters under methanogenic conditions. *Environ Sci Technol* 31:2761-2764
59. Elder, D. J. E., Kelly, D. J., 1994. The bacterial degradation of benzoic-acid and benzenoid compounds under anaerobic conditions—unifying trends and new perspectives. *FEMS Microbiol Rev* 13:441-468
60. El-Hadj, T. B., Dosta, J., Mata-Alvarez, J., 2006. Biodegradation of PAH and DEHP micro-pollutants in mesophilic and thermophilic anaerobic sewage sludge digestion. *Water Sci Technol* 53:99-107
61. Emerson, C., 2010. Australia Bans Phthalates in Toys, Dr C. Emerson, ALP. http://www.google.com/search?hl=&q=Australia+Bans+Phthalates+in+Toys&sourceid=navclient-ff&rlz=1B3WZPB_enCN376CN377&ie=UTF-8
62. Engel, S. M., Miodovnik, A., Canfield, R. L., Zhu, C., Silva, M. J., Calafat, A. M., Wolff, M. S., 2010. Prenatal Phthalate Exposure is Associated with Childhood Behavior and Executive Functioning. *Environ. Health Perspect.* 2010 Jan. 8. [Epub ahead of print].

63. Engelhardt, G., Tillmanns, G., Wallnofer, P. R., Hutzinger, O., 1977. Biodegradation of di-iso-butyl phthalate and related dialkyl phthalates by *Penicillium lilacinum*. *Chemosphere* 6:347–354.
64. European Union (EU), 1999. Ban of phthalates in childcare articles and toys, press release IP/99/829, 10 November 1999.
65. European Union Risk Assessment Report (EU-RAR), 2003. Dibutyl Phthalate. Addendum to the Environmental Section. CAS No: 84-74-2. EINECS No: 201-557-4. P40.
66. Evans, W. C., 1997. Biochemistry of the bacterial catabolism of aromatic compounds in aerobic environments. *Nature* 270:17-22.
67. Fattom, A., Shillo, M. 1984. Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. *Appl. Environ. Microbiol.* 47(1):135-143.
68. Fauser, P., Vikelsøe, J., Sorensen, P. B., Carlsen, L., 2003. Phthalates, nonylphenols and LAS in an alternately operated wastewater treatment plant—fate modelling based on measured concentrations in wastewater and sludge. *Water Res* 37:1288–1295
69. Field, J. A., Reed, R. L., 1996. Nonylphenol Polyethoxy Carboxylate Metabolites of Nonionic Surfactants in US. Paper Mill Effluents, Municipal Sewage Treatment Plant Effluents, and River Waters. *Environ. Sci. Technol.* 30:3544-3550.
70. Ganji, S. H., Karigar, C. S., Pujar, B. G., 1995. Metabolism of dimethylterephthalate by *Aspergillus niger*. *Biodegradation* 6:61–66
71. Gavala, H. N., Alatrisme-Mondragon, F., Iranpour, R., Ahring, B. K., 2003. Biodegradation of phthalate esters during the mesophilic anaerobic digestion of sludge. *Chemosphere* 52:673–682
72. Gibbons, J. A., Alexander, M., 1989. Microbial degradation of sparingly soluble organic chemicals: phthalate esters. *Environmental Toxicology and Chemistry* 8:283-29
73. GovTrack.US, 2008. H.R. 4040--110th Congress. Consumer Product Safety Improvement Act of 2008, GovTrack.us (database of federal legislation) (accessed 25 December 2009).
74. GreenFacts 2009. Phthalates in school supplies. Website:<http://copublications.greenfacts.org/en/phthalates-school-supplies/>. Retrieved 2010-03-10.
75. Grifoll, M., Selifonov, S. A., Chapman, P. J., 1994. Evidence for a novel pathway in the degradation of fluorene by *Pseudomonas* sp strain F274. *Appl Environ Microbiol* 60:2438–2449
76. Groshart, C., Okkerman, P. C., 2000. Toward the establishment of a priority list of substances for further evaluation of their role in endocrine disruption: Preparation of a candidate list of substances as a basis for priority setting. Final report (incorporating corrigenda to final report dated 21 June, 2000). European Commission DG Env.
77. Gu, J. D., Li, J., Wang, Y., 2005. Biochemical pathway and degradation of phthalate ester isomers by bacteria. *Water Sci Technol* 52:241–248.
78. Guenther, K., Heinke, V., Thiele, B., Kleist, E., Prast, H., Raecker, T., 2002. Endocrine disrupting nonylphenols are ubiquitous in food. *Environ. Sci. Technol.* 36:1676-1680.
79. Guerin, W. F., Jones, G. E., 1988. Two-stage mineralization of phenanthrene by estuarine enrichment cultures. *Appl Environ Microbiol* 54:929–936.
80. Hall, D. O., Hawkins, S. E., 1975. Laboratory Manual of Cell Biology. The English University Press, London (Chapter 2).
81. Hallmark, N., Walker, M., McKinnell, C., et al. 2007. Effects of monobutyl and di(n-butyl) phthalate in vitro on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects in vivo in the fetal rat and neonatal marmoset and in vitro in the human. *Environ. Health Perspect.* 115 (3):390–6.
82. Hashimoto, M., Takahashi, K., 1982. Calorimetric studies of microbial growth: quantitative relation between growth thermograms and inoculum size. *Agr. Biol. Chem.* 46:1559-1564.
83. Hashizume, K., Nanya, J., Toda, C., Yasui, T., Nagano, H., Kojima, N., 2002. Phthalate esters detected in various water samples and biodegradation of the phthalates by microbes isolated from river water. *Biol Pharm Bull* 25(2):209–214
84. Hauser, R., Duty, S., Godfrey-Bailey, L., Calafat, A. M., 2004. Medications as a source of human exposure to phthalates. *Environmental Health Perspectives* 112(6):751-753.
85. Herbert, H. P., Zeng, F. H., 2004. Adsorption of phthalates by activated sludge and its biopolymers, *Environ. Technol.* 25(7):757–761.
86. Hernández-Díaz, S., Mitchell, A. A., Kelley, K. E., Calafat, A. M., Hauser, R., 2009. Medication as a potential source of exposure to phthalates in the US population. *Environ. Health Perspect.* 117 (2):185–9.

87. Heudorf, U., Mersch-Sundermann, V., Angerer, J., 2007. Phthalates: toxicology and exposure. *Int. J. Hyg. Environ. Health* 210(5):623–634.
88. Hileman, B., 2007. California Bans Phthalates in Toys for Children. *Chemical and Engineering News*, 12.
89. Hojslev, P. J., Breindahal, T., 2000. Plasticizers in total diet samples, Baby Food and Infant Formulae. *Food Additives and Contaminants*, 17:133-141.
90. Horn, O., Nalli, S., Cooper, D., Nicell, J., 2004. Plasticizer metabolites in the environment. *Water Res* 38:3693–3698
91. Huber, W.W., Grasl-Kraupp, B., Schulte-Hermann, R., 1996. Hepatocarcinogenic potential of di(2-ethylhexyl)phthalate in rodents and its implications on human risk. *Crit Rev Toxicol* 26 (4): 365–481.
92. Hylland, K., Haux, C., 1997. Effects of environmental oestrogens on marine fish species. *Trends in Analytical Chemistry (TrAC)* 6(10):606-612..
93. Ishikawa, K., Sato, M., Yoshida, T., 1981. Expression of rat heme oxygenase in *Escherichia coli* as a catalytically active, full-length form that binds to bacterial membranes. *Eur J Biochem.* 202(1):161-165
94. Jackson, M. A., Labeda, D. P., Becker, L. A., 1996. Isolation for bacteria and fungi for the hydrolysis of phthalate and terephthalate esters. *J Ind Microbiol* 16:301–304
95. Jain, A., Nishad, K. K., Bhosle, N. B., 2007. Effects of DNP on cell surface properties of marine bacteria and its implication for adhesion to surfaces. *Biofouling* 23, 171-177.
96. Jianlong, W., Ping, L., Yi, Q., 1995. Microbial degradation of di-n-butyl phthalate. *Chemosphere* 31(9):4051-4056.
97. Jianlong, W., Ping, L., Yi, Q., 1996. Biodegradation of phthalic acid esters by acclimated activated sludge. *Environ. Int.* 22:737-741.
98. Jianlong, W., Ping, L., Yi, Q., 2004. Biodegradation of phthalic acid esters (PAE's) in soil bioaugmented with acclimated activated sludge, *Process Biochem.* 39:1837–1841.
99. Johnson, B. T., Heitkamp, M. A., 1984. Environmental and chemical factors influencing the biodegradation of phthalic acid esters in freshwater sediments. *Environmental Pollution* 8:101-118.
100. Jonsson, S., Ejlertsson, J., Svensson, B. H., 2003a. Behaviour of mono- and diesters of o-phthalic acid in leachates released during digestion of municipal solid waste under landfill conditions. *Adv Environ Res* 7:429–440
101. Jonsson, S., Ejlertsson, J., Svensson, B. H., 2003b. Transformation of phthalates in young landfill cells. *Waste Management* 23:641–651
102. Jonsson, S., Ejlertsson, M., Ledin, A., Mersiowsky, I., Svensson, B. H., 2003c. Mono- and diesters from o-phthalic acid in leachates from different European landfills. *Water Res* 37:609–617
103. Juneson, C., Ward, O. P., Singh, A., 2001. Biodegradation of bis-(2-ethylhexyl) phthalate in a soil slurry-sequencing batch reactor. *Process Biochem* 37:305–313
104. Juneson, C., Ward, O. P., Singh, A., 2002. Biodegradation of dimethyl phthalate with high removal rates in a packed-bed reactor. *World J Microbiol Biotechnol* 18:7–10
105. Kaiser, J., 2005. Toxicology. Panel finds no proof that phthalates harm infant reproductive systems. *Science* 310 (5747):422.
106. Karplus, P. A., 1997. Hydrophobicity regained. *Protein Science.* 6:1302-1307.
107. Kauzmann, W., 1959. Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* 14:1-63.
108. Kawabata, T., Yamano, H., Takahashi, K., 1983. An attempt to characterize calorimetrically the inhibitory effect of foreign substances on microbial degradation of glucose in soil. *Agric. Biol. Chem.* 47:1281-1288.
109. Keith, L. H., Telliard, W. A., 1999. Priority Pollutants I: a perspective view. *Environmental Science and Technology* 13:416-423.
110. Keyser, P., Pujar, B. G., Eaton, R. W., Ribbons, D. W., 1976. Biodegradation of phthalates and their esters by bacteria. *Environ Health Perspect* 18:159–166
111. Kim, Y. H., Lee, J., 2005. Enzymatic degradation of dibutyl phthalate and toxicity of its degradation products. *Biotechnol Lett* 27:635–639
112. Kim, Y. H., Lee, J. W., Ahn, J. Y., Gu, M. B., Moon, S. H., 2002. Enhanced degradation of an endocrine-disrupting chemical, butyl benzyl phthalate, by *Fusarium oxysporum* f. sp pisi cutinase. *Appl Environ Microbiol* 68:4684–4688
113. Kim, Y. H., Lee, J., Moon, S. H., 2003. Degradation of an endocrine disrupting chemical, DEHP [di-(2-ethylhexyl)-phthalate], by *Fusarium oxysporum* f. sp pisi cutinase. *Appl Microbiol Biotechnol* 63:75–80

114. Kim, Y. H., Min, J. H., Bae, K. D., Gu, M. B., Lee, J. 2005. Biodegradation of dipropyl phthalate and toxicity of its degradation products: a comparison of *Fusarium oxysporum* f. sp *pisi* cutinase and *Candida cylindracea* esterase. *Archives of Microbiol* 184:25–31
115. Kim, Y. H., Seo, H. S., Min, J., Kim, Y. C., Ban, Y. H., Han, K. Y., Park, J. S., Bae, K. D., Gu, M. B., Lee, J., 2007. Enhanced degradation and toxicity reduction of dihexyl phthalate by *Fusarium oxysporum* f. sp *pisi* cutinase. *J Appl Microbiol* 102:221–228
116. Kim, D. S., Um, H.-J., Lim, E.-S., Min, J., Kim, Y. H. 2008. Degradation of diphenyl phthalate by *Sphingomonas chungbukensis*. *Biotechnol Lett* 30:93–96
117. Kim, H. T., Kim, K. P., Uchiki, T., Gygi, S. P., Goldberg, A. L., 2009. S5a promotes protein degradation by blocking synthesis of nondegradable forked ubiquitin chains. *EMBO J* 28:1867–1877
118. Kimura, T., Takahashi, K., 1985. Calorimetric studies of soil microbes: quantitative relation between heat evolution during microbial degradation of glucose and changes in microbial activity in soil. *J. Gen. Microbiol.* 131:3083–3089.
119. Kiyohara, H., Nagao, K., 1978. Catabolism of phenanthrene and naphthalene by bacteria. *J Gen Microbiol* 105:69–75
120. Kleerebezem, R., Mortier, J., Pol, L. W. H., Lettinga, G., 1997. Anaerobic pre-treatment of petrochemical effluents: terephthalic acid wastewater. *Water Sci Technol* 36:237–248
121. Kleerebezem, R., Pol, L. W. H., Lettinga, G., 1999. Anaerobic biodegradability of phthalic acid isomers and related compounds. *Biodegradation* 10:63–73.
122. Kleerebezem, R., Ivala, M., Pol, L. W. H., Lettinga, G., 1999a. High-rate treatment of terephthalate in anaerobic hybrid reactors. *Biotechnol Prog* 15:347–357
123. Kleerebezem, R., Pol, L. W. H., Lettinga, G., 1999b. Anaerobic biodegradability of phthalic acid isomers and related compounds. *Biodegradation* 10:63–73
124. Kleerebezem, R., Pol, L. W. H., Lettinga, G., 1999c. Energetics of product formation during anaerobic degradation of phthalate isomers and benzoate. *FEMS Microbiol Ecology* 29:273–282
125. Kleerebezem, R., Beckers, J., Pol, L. W. H., Lettinga, G., 2005. High rate treatment of terephthalic acid production wastewater in a two-stage anaerobic bioreactor. *Biotechnol Bioeng* 91:169–179
126. Kolarik, B., Bornehag, C., Naydenov, K., Sundell, J., Stavova, P., Nielsen, O., 2008. The concentration of phthalates in settled dust in Bulgarian homes in relation to building characteristic and cleaning habits in the family. *Atmospheric Environment* 42 (37): 8553–9.
127. Kormelink, F. J. M., Lefebvre, B., Strozyk, F., Voragen, A. G. J., 1993. The purification and characterisation of an acetyl xylan esterase from *Aspergillus niger*. *J. Biotechnol.* 27:267–282.
128. Kuntiya, A., Nicolella, C., Pyle, L., Poosaran, N., 2005. Effect of sodium chloride on cell surface hydrophobicity and formation of biofilm in membrane bioreactor. *Songklanakarin J. Sci. Technol.* 27(5):1073–1082.
129. Kurane, R., Suzuki, T., Takahara, Y., 1978. Microbial degradation of phthalate esters, *Agr. Biol. Chem.* 42:1469–1473.
130. Lee, S. M., Koo, B. W., Lee, S. S., Kim, M. K., Choi, D. H., Hong, E. J., Jeung, E. B., Choi, I. G., 2004. Biodegradation of dibutylphthalate by white rot fungi and evaluation on its estrogenic activity. *Enzyme Microb Technol* 35:417–423
131. Lee, S. M., Lee, J. W., Koo, B. W., Kim, M. K., Choi, D. H., Choi, I. G., 2007. Dibutyl phthalate biodegradation by the white rot fungus, *Polyporus brumalis*. *Biotechnol Bioeng* 97:1516–1522
132. Li, B., Chi, J., Wu, W. X., Wang, Z. K., 2007. Effect of nutrients and light on biodegradation of dibutyl phthalate and di-2-ethylexyl phthalate in haihe estuary. *Bull Environ Contam Toxicol* 79:80–83
133. Liang, D. -W., Zhang, T., Fang, H. H. P., 2007. Anaerobic degradation of dimethyl phthalate in wastewater in a UASB reactor. *Water Res* 41:2879–2884
134. Liang, D. -W., Zhang, T., Fang, H. H. P., He, J., 2008. Phthalates biodegradation in the environment. *Appl Microbiol Biotechnol* 80:183–198.
135. Liu, S. M., Chi, W. C., 2003. CO₂-H₂ dependent anaerobic biotransformation of phthalic acid isomers in sediment slurries. *Chemosphere* 52:951–958
136. Ljungh, Å., Hjertén, S., Wadström, T., 1985. High surface hydrophobicity of autoaggregating *Staphylococcus aureus* isolated from human infections studied with the salt aggregation test. *Am. Soc. Microbiol.* 47:522–526.

137. Lomenick, J. P., Calafat, A. M., Melguizo, Castro, M. S., Mier, R., Stenger, P., Foster M. B., Wintergerst, K. A., 2009. "Phthalate Exposure and Precocious Puberty in Females". *The Journal of Pediatrics* 156(2):221–5. [http://linkinghub.elsevier.com/retrieve/pii/S0022-3476\(09\)00927-5](http://linkinghub.elsevier.com/retrieve/pii/S0022-3476(09)00927-5). "Exposure is not associated with precocious puberty in female children".
138. Lovrien, R., Mark, E., Ferry, L., Magnuson, T. S., Blanchette, R. A., 1989. Microbial calorimetric analysis. Lignin-related compounds in micromolar concentrations. *ACS Symposium Series*, Vol. 399, Chapter 39, pp 544–558; Chapter DOI: 10.1021/bk-1989-0399.ch039. ISBN13: 9780841216587eISBN: 9780841212503. Publication Date (Print): July 31, 1989. Copyright © 1989 American Chemical Society.
139. Luong, J. H. T., Volesky, B., 1980. Determination of the Heat of Some Aerobic Fermentations. *Can J Chem Eng* 58:497
140. Ma, J., Qi, W. T., Yang, L. N., Yu, W. T., Xie, Y. B., Wang, W., Ma, X. J., Xu, F., Sun, L. X., 2007. Microcalorimetric study on the growth and metabolism of microencapsulated microbial cell culture. *J. Microbiol. Methods* 68:172.
141. Mackintosh, C.E., Maldonado, J.A., Ikonomou, M.G., Gobas, F.A.P.C., 2006. Sorption of phthalate esters and PCBs in marine ecosystem, *Environ. Sci. Technol.* 40:3481-3488
142. Madsen, P. L., Thyme, J. B., Henriksen, K., Moldrup, P., Roslev, P., 1999. Kinetics of di-(2-ethylhexyl) phthalate mineralization in sludge amended soil. *Environ Sci Technol* 33:2601–2606.
143. Main, K. M., Mortensen, G. K., Kaleva, M. M., Boisen, K. A., Damgaard, I. N., Chellakooty, M., Schmidt, I. M., Suomi A.-M., Virtanen, H. E., Petersen, J. H., Andersson, A.-M., Toppari, J., Skakkebaek, N. E., 2006. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environ Health Perspect* 114:270-276.
144. Marshall, K.C., 1991. The importance of studying microbial cell surfactants. In: Mozes N (ed) Microbial cell surface analysis, 1st edn. Wiley-VCH Publishers, New York
145. Marttinen, S. K., Kettunen, R. H., Rintala, J. A., 2003. Occurrence and removal of organic pollutants in sewages and landfill leachates. *Sci Total Environ* 301:1–12
146. Marttinen, S. K., Hanninen, K., Rintala, J. A., 2004a. Removal of DEHP in composting and aeration of sewage sludge. *Chemosphere* 54:265–272
147. Marttinen, S. K., Ruissalo, M., Rintala, J. A., 2004b. Removal of bis-(2-ethylhexyl) phthalate from reject water in a nitrogen-removing sequencing batch reactor. *J Environ Manage* 73:103–109
148. Maruyama, K., Akita, K., Naitou, C., Yoshida, M., Kitamura, T., 2005. Purification and characterization of an esterase hydrolyzing monoalkyl phthalates from *Micrococcus* sp YGJ1. *J Biochem* 137:27–32
149. Matsumoto, M., Hirata-Koizumi, M., Ema, M., 2008. Potential adverse effects of phthalic acid esters on human health: a review of recent studies on reproduction. *Regul Toxicol Pharm* 50:37–49
150. Medrzycka, K.B., 1991. The effect of particle concentration in extremely dilute solutions. *Prog Colloid Polym Sci.* 269:85- 90.
151. Miyoshi, M., 1895. Die durchbohrung von membranen durch pilzfäden. *Jahrb Wiss Bot* 28:269-289.
152. Mohan, S. V., Shailaja, S., Krishna, M. R., Reddy, K. B., Sarma, P. N., 2006. Bioslurry phase degradation of di-ethyl phthalate (DEP) contaminated soil in periodic discontinuous mode operation: influence of bioaugmentation and substrate partition. *Process Biochem* 41:644–652
153. Moore, N. P., 2000. The estrogenic potential of the phthalate esters. *Repro. Toxicol.* 14:183-192
154. Nakazawa, T., Hayashi, E., 1977. Phthalate metabolism in *Pseudomonas testosterone*—accumulation of 4,5-dihydroxyphthalate by a mutant strain. *J Bacteriol* 131:42–48
155. Naumova, R. P., Zaripova, S. K., Usmanova, L. P., 1986. Regulation of terephthalate catabolism in *Rhodococcus rubropertinctus*. *Microbiology* 55:729–734
156. Nomura, Y., Nakagawa, M., Ogawa, N., Harashima, S., Oshima, Y., 1992. Genes in *pht* plasmid encoding the initial degradation pathway of phthalate in *Pseudomonas putida*. *J Ferm Bioeng* 74:333–344
157. Nozawa, T., Maruyama, Y., 1988. Anaerobic metabolism of phthalate and other aromatic-compounds by a denitrifying bacterium. *J Bacteriol* 170:5778–5784
158. Nuñez, L., Barros, N., Barja, I., 1994. A kinetic analysis of the degradation of glucose by soil microorganisms studied by microcalorimetry. *Thermochim. Acta* 237:73-81.
159. Núñez-Regueiler, L., Rodríguez-Añón, J. A., Proupín-Castiñeras, J., Núñez-Fernández, O., 2006. Microcalorimetric study of changes in the microbial activity in humic cambisol after reforestation with eucalyptus in Galicia (NW Spain). *Soil Biol. Biochem.* 38:115.

160. O'Connor, O. A., Rivera, M. D., Young, L. Y., 1989. Toxicity and biodegradation of phthalic-acid esters under methanogenic conditions. *Environ Toxicol Chem* 8:569–576
161. O'Grady, D. P., Howard, P. H., Werner, A. F., 1985. Activated sludge biodegradation of 12 commercial phthalate esters. *Applied Environmental Microbiology* 49:433–445.
162. Oliver, R., May, E., Williams, J., 2005. The occurrence and removal of phthalates in a trickle filter STW. *Water Res* 39:4436–4444
163. Oliver, R., May, E., Williams, J., 2007. Microcosm investigations of phthalate behaviour in sewage treatment biofilms. *Sci Total Environ* 372:605–614
164. Olivera, N. L., Commendatore, M. G., Delgado, O., Esteves, J. L., 2003. Microbial characterization and hydrocarbon biodegradation potential of natural bilge waste microflora. *J. Ind. Microbiol. Biotechnol.* 30:542–548.
165. Painter, S. E., Jones, W. J., 1990. Anaerobic bioconversion of phthalic acid esters by natural inocula. *Environ. Technol.* 11:1015–1026.
166. Pan, W.-H., Li, P.-L., Liu, Z., 2006. The correlation between surface hydrophobicity and adherence of *Bifidobacterium* strains from centenarians' faeces. *Food microbiology. Anaerobes* 12:148–152.
167. Papagianni, M., Matthey, M., Kristiansen, B., 1999. Citric acid production and morphology of *Aspergillus niger* as functions of the mixing intensity in a stirred tank and a tubular loop bioreactor. *Biochem. Eng. J.* 2(3):197–205.
168. Petrovic, M., Barceló, D., 2000. Determination of Anionic and Nonionic Surfactants, Their Degradation Products, and Endocrine-Disrupting Compounds in Sewage Sludge by Liquid Chromatography/Mass Spectrometry. *Analytical Chemistry* 72(19):4560.
169. Postellon, D. C., 2008. Baby Care Products (letter). *Pediatrics* 121 (6): 1292. PMID 18519505. <http://pediatrics.aappublications.org/cgi/content/full/121/6/1292>. Retrieved 2009-12-14.
170. Prabhu, Y., Phale, P. S., 2003. Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: Novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. *Appl. Microbiol. Biotechnol.* 61:342–351.
171. Prado, A. G. S., Airoidi, C., 1999. The influence of moisture on microbial activity of soils. *Thermochim. Acta.* 332:71.
172. Prado, A.G.S., Airoidi, C., 2000. Effects of the pesticide 2,4-D on microbial activity of the soil monitored by microcalorimetry. *Thermochim. Acta* 349:17–22.
173. Pye, V. I., Patrick, R., 1983. Ground water contamination in the United States. *Science* 221:713–718.
174. Qiu, Y. L., Sekiguchi, Y., Imachi, H., Kamagata, Y., Tseng, I. C., Cheng, S. S., Ohashi, A., Harada, H., 2004. Identification and isolation of anaerobic, syntrophic phthalate isomer-degrading microbes from methanogenic sludges treating wastewater from terephthalate manufacturing. *Appl Environ Microbiol* 70:1617–1626
175. Raihan, S., Ahmed, N., Ali, R., Khan, N., Eshaq, A., 1992. Production of exopolysaccharide by an indigenous soil isolates. *JIAS*. 5(4). <http://www.medicaljournal-ias-org/5-4/Raihan.html>
176. Rais-Bahrami, K., Nunez, S., Revenis, M. E., Luban, N. L., Short, B. L., 2004. Follow-up study of adolescents exposed to di(2-ethylhexyl) phthalate (DEHP) as neonates on extracorporeal membrane oxygenation (ECMO) support. *Environ. Health Perspect.* 112 (13): 1339–1340.
177. Reddy, P. G., Singh, H. D., Pathak, M. G., Bhagat, S. D., Baruah, J. N., 1983. Isolation and functional characterization of hydrocarbon emulsifying and solubilizing factors produced by a *Pseudomonas* species. *Biotechnol. Bioeng.* 25:387–401.
178. Rivas A., Olea, N., Olea-Serrano, F., 1997. Human exposure to endocrine-disrupting chemicals: assessing the total estrogenic xenobiotic burden. *Trends in Analytical Chemistry (TrAC)* 16(10):613.
179. Romano-Riquer, S. P., Hernández-Avila, M., Gladen, B. C., Cupul-Uicab, L. A., Longnecker, M. P., 2007. Reliability and determinants of anogenital distance and penis dimensions in male newborns from Chiapas, Mexico. *Paediatr Perinat Epidemiol* 21 (3): 219–28.
180. Romero, H. E., Sumanasekera, G. U., Mahan, G. D., Eklund, P. C., 2002. Thermoelectric power of single-walled carbon nanotube films. *Phys. Rev. B* 65(20) 6 pp
181. Rosenberg, E., Kaplan, N., Pines, O., Rosenberg, M., Gutnick, D., 1983. Capsular polysaccharides interfere with adherence of *Acinetobacter calcoaceticus* to hydrocarbon. *FEMS Microbiol. Lett.* 17:157–160.
182. Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E., Ron, E. Z., 1992. Petroleum bioremediation – a multiphase problem. *Biodegradation* 3:337–350.

183. Roslev, P., Vorkamp, K., Aarup, J., Frederiksen, K., Nielsen, P. H., 2007. Degradation of phthalate esters in an activated sludge wastewater treatment plant. *Water Res* 41:969-976
184. Roslev, P., Madsen, P. L., Thyme, J. B., Henriksen, K., 1998. Degradation of phthalate and di-(2-ethylhexyl) phthalate by indigenous and inoculated microorganisms in sludge-amended soil. *Appl Environ Microbiol* 64:4711-4719
185. Rudel, R., Perovich, L., 2008. Endocrine disrupting chemicals in indoor and outdoor air. *Atmospheric Environment* 43 (1): 170-81.
186. Salazar-Martinez, E., Romano-Riquer, P., Yanez-Marquez, E., Longnecker, M. P., Hernandez-Avila, M., 2004. Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. *Environ. Health*. 3 (1): 8. Retrieved 2010-03-20
187. Sathyanarayana, S., Karr, C. J., Lozano, P., *et al.*, 2008. Baby care products: possible sources of infant phthalate exposure. *Pediatrics* 121 (2): e260-8
188. Schlafli, H. R., Weiss, M. A., Leisinger, T., Cook, A. M., 1994. Terephthalate 1,2-dioxygenase system from *comamonas-testosteroni-t-2*—purification and some properties of the oxygenase component. *J Bacteriol* 176:6644-6652
189. Schwarzbauer, J., Heim, S., Brinker, S., Littke, R., 2002. Occurrence and alteration of organic contaminants in seepage and leakage water from a waste deposit landfill. *Water Res* 36:2275-2287
190. Shailaja, S., Ramakrishna, M., Mohan, S. V., Sarma, P. N., 2007. Biodegradation of di-n-butyl phthalate (DnBP) in bioaugmented bioslurry phase reactor. *Bioresour Technol* 98:1561-1566
191. Shelton, D. R., Boyd, S. D., Tiedje, J. M., 1984. Anaerobic biodegradation of phthalic-acid esters in sludge. *Environ Sci Technol* 18:93-97
192. Singh, S., Fett, W. F., 1995. Stimulation of exopolysaccharide production by fluorescent pseudomonads in sucrose media due to dehydration and increased osmolarity. *FEMS Microbiol. Lett.* 130:301-306.
193. Sivamurthy, K., Swamy, B. M., Pujar, B. G., 1991. Transformation of dimethylterephthalate by the fungus *Sclerotium rolfsii*. *FEMS Microbiol Lett* 79:37-40
194. Sparling, G. P., 1983. Estimation of microbial biomass and activity in soil using microcalorimetry. *Journal of Soil Science* 34:381-390.
195. Stahlhut, R. W., van Wijngaarden, E., Dye, T. D., Cook, S., Swan, S. H., 2007. Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult U.S. males. *Environ. Health Perspect.* 115 (6): 876-82.
196. Staples, C. A., Peterson, D. R., Parkerton, T. F., Adams, W. J., 1997. The environmental fate of phthalate esters: a literature review. *Chemosphere* 35:667-749
197. Sugatt, R. H., Ogrady, D. P., Banerjee, S., Howard, P. H., Gledhill, W. E., 1984. Shake flask biodegradation of 14 commercial phthalate esters. *Appl Environ Microbiol* 47:601-606
198. Summerfield, W., Goodson, A., Cooper, I., 1998. *Food Additives and Contaminants*. 1517:818.
199. Swan, H., Piippo, K., Viitasalo, M., Heikkila, P., Paavonen, T., Kainulainen, K., Kere, J., Keto, P., Kontula, K., Toivonen, L., 1999. Arrhythmic disorder mapped to chromosome 1q42-q43 causes malignant polymorphic ventricular tachycardia in structurally normal hearts. *J Am Coll Cardiol.* 34:2035-2042.
200. Swan, S. H., Main, K. M., Liu, F., Stewart, S. L., Kruse, R. L., Calafat, A. M., Mao, C. S., Redmon, J. B., Ternand, C., L., Sullivan, S., Teague, J. L., 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ. Health Perspect.* 113(8):1056-61. <http://ehpnet1.niehs.nih.gov/members/2005/8100/8100.html>.
201. Tahmourespour, A., Kermanshahi, R. K., Salehi, R., Nabinejad, A., 2008. The relationship between cell surface hydrophobicity and antibiotic resistance of Streptococcal strains isolated from dental plaque and caries. *Iranian Journal of Basic Medical Sciences* 10:251-255.
202. Tan, A. M., Huang, Y. Q., Qu, S. S., 1999. Determination of the respiratory burst of polymorphonuclear leukocytes by microcalorimetry. *J Biochem Biophys Methods* 37:91-94.
203. Teo, K. C., Xu, H. L., Tay, J. H., 1998. Molecular mechanism of granulation. II: Proton translocation activity. *J. Environ. Eng.* 126:411- 418.
204. Tiehm, A., 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. *Appl. Environ. Microbiol.* 60:258-263.
205. Tilson, H. A., 2008. Environmental Health Perspectives (EHP) Papers of the Year, 2008. *Environ. Health Perspect.* 116 (6): A234-A234. doi:10.1289/ehp.11684.
206. Toppari, J., Virtanen, H., Skakkebaek, N. E., Main, K. M., 2006. Environmental effects on hormonal regulation of testicular descent. *J Steroid Biochem Mol Biol* 102 (1-5): 184-6.

207. Tur, M. Y., Huang, J. C., 1997. Treatment of phthalic waste by anaerobic hybrid reactor. *J Environ. Eng-ASCE* 123:1093-1099
208. Turner, A., Rawling, M. C., 2000. The behaviour of di-(2-ethylhexyl) phthalate in estuaries. *Marine Chem.* 68:203-217
209. United State CDC. 2005. Third National Report on Human Exposure to Environmental Chemicals, U.S. CDC.
210. United States- Environmental Protection Agency (US-EPA), 1982. Fate of priority pollutants in publicly owned treatment works. Final Report EPA 440/1-82/303.
211. Vega, D., Bastide, J., 2003. Dimethylphthalate hydrolysis by specific microbial esterase. *Chemosphere* 51:663-668
212. Wadsö, I., 1997. Isothermal microcalorimetry near ambient temperature: An overview and discussion. *Thermochim. Acta* 294:1-11.
213. Wadsö, I., 1980. Some problems in calorimetry measurements on cellular systems. In: Beezer, A. E., (Ed.), *Biological Microcalorimetry*. Academic Press, London, pp. 247-274.
214. Wang, F., Yao, J., Tian, L., Zhou, Y., Chen, H.-L., Chen, H.-Y., Gai, N., Chen, Y.-J., Zhuang, R.-S., Zaray, G., Maskow, T., Bramanti, E., 2008. Microcalorimetric investigation of the toxic action of ammonium ferric(III) sulfate on the metabolic activity of pure microbes. *Environmental Toxicology and Pharmacology* 25:351-357.
215. Wang, J., Liu, P., Qian, Y., 1995. Microbial degradation of di-n-butyl phthalate. *Chemosphere* 31:4051-4056.
216. Wang, Y. Z., Zhou, Y. M., Zystra, G. J., 1997. Molecular analysis of isophthalate and terephthalate degradation by *Comamonas testosteroni* YZW-D. *Environ Health Perspect* 103:9-12
217. Wang, J. L., Liu, P., Shi, H. C., Qian, Y., 1997a. Biodegradation of phthalic acid ester in soil by indigenous and introduced microorganisms. *Chemosphere* 35:1747-1754
218. Wang, J. L., Liu, P., Shi, H. C., Qian, Y., 1997b. Kinetics of phthalic acid ester degradation by acclimated activated sludge. *Process Biochem* 32:567-571
219. Wang, J. L., Chen, L. J., Shi, H. C., Qian, Y., 2000. Microbial degradation of phthalic acid esters under anaerobic digestion of sludge. *Chemosphere* 41:1245-1248
220. Wang, J. L., Xuan, Z., Wu, W. Z., 2004a. Biodegradation of phthalic acid esters (PAEs) in soil bioaugmented with acclimated activated sludge. *Process Biochem* 39:1837-1841
221. Wang, S. G., Lin, X. G., Yin, R., Hou, Y. L., 2004b. Effect of inoculation with arbuscular mycorrhizal fungi on the degradation of DEHP in soil. *J Environ Sci China* 16:458-461
222. Wang, Y. Y., Fan, Y. Z., Gu, J. D. 2004c. Dimethyl phthalate ester degradation by two planktonic and immobilized bacterial consortia. *Int Biodeter Biodegr* 53:93-101
223. Wensing, M., Uhde, E., Salthammer, T., 2005. Plastics additives in the indoor environment- flame retardants and plasticizers. *Sci Total Environ* 339:19-40
224. Wolfe, N. L., Steen, W. C., Burns, L. A., 1980. Phthalate ester hydrolysis—linear free energy relationships. *Chemosphere* 9:403-408
225. Wrangstadh, M., Conway, P. L., Kjellberg, S., 1986. The production and release of an extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. and the effect thereof on adhesion. *Arch. Microbiol.* 145:220-227.
226. Wu, J. H., Liu, W. T., Tseng, I. C., Cheng, S. S., 2001. Characterization of microbial consortia in a terephthalate-degrading anaerobic granular sludge system. *Microbiology* 147:373-382
227. Xia, F., Zheng, P. Z. Q., Feng, X., 2004. Relationship between quantitative structure and biodegradability for phthalic acid ester compounds. *J Zhejiang Univ (Agri Life Sci)* 30:141-146
228. Xu, X. R., Li, H. B., Gu, J. D., 2005. Biodegradation of an endocrine-disrupting chemical di-n-butyl phthalate ester by *Pseudomonas fluorescens* B-1. *Int Biodeter Biodegr* 55:9-15
229. Yamano, H., Takahashi, K., 1983. Temperature effect on the activity of soil microbes measured from heat evolution during the degradation of several carbon sources. *Agricultural and Biological Chemistry* 47, 1493-1499.
230. Yan, H., Pan, G., 2004. Increase in biodegradation of dimethyl phthalate by *Closterium lunula* using inorganic carbon. *Chemosphere* 55:1281-1285
231. Yan, H., Pan, G., Liang, P. L., 2002. Effect and mechanism of inorganic carbon on the biodegradation of dimethyl phthalate by *Chlorella pyrenoidosa*. *J Environ Sci Health Part A* 37:553-562
232. Yao, J., Xu, C.Q., Wang, F., Tian, L., Wang, Y.X., Chen, H.L., Zhou, Y., Choi, M. M. F., Bramanti, E., Maskow, T., 2007. An in vitro microcalorimetric method for studying the toxic effect of cadmium on microbial activity of an agricultural soil, *Ecotoxicology* 16:503-509.

233. Yao, J., Tian, L., Wang, Y., Djah, A., Wang, F., Chen, H.-L., Su, C.-L., Zhuang, R., Zhou, Y., Choi, M.M.F., Bramanti, E., 2008. Microcalorimetric study of the toxic effect of hexavalent chromium on microbial activity of Wuhan brown sandy soil: An in vitro approach. *Ecotox. Environ. Safe.* 69:289-295.
234. Yuan, S. Y., Liu, C., Liao, C. S., Chang, B. V., 2002. Occurrence and microbial degradation of phthalate esters in Taiwan river sediments. *Chemosphere* 49(10):1295–1299
235. Yuwatini, E., Hata, N., Taguchi, S., 2006. Behavior of di-(2-ethylhexyl) phthalate discharged from domestic waste water into aquatic environment. *J Environ Monitor* 8:191–196
236. Zeng, X. B., Ungar, G., Spells, S. J., Brooke, G. M., Farren, C., Harden, A., 2003. Crystal-amorphous polymer interface studied by neutron and X-Ray scattering on labeled binary ultralong alkanes. *Phys. Rev. Lett.* 90, 155508 [4 pages]
237. Zeng, F., Cui, K., Li, X., Fu, J., Sheng, G., 2004. Biodegradation kinetics of phthalate esters by *Pseudomonas fluorescens* FS1. *Process Biochem.* 39:1125-1129.
238. Zeng, F., Cui, K. Y., Xie, Z., Wu, L. N., Liu, M., Song, G. Q., Lin, Y. J., Luo, D. L., Zeng, Z. X., 2008. Phthalate esters (PAEs): emerging organic contaminants in agricultural soils in peri-urban areas around Guangzhou, China, *Environ. Pollut.* 158(2):425-434
239. Zheng, D., Liu, Y., Zhang, Y., Chen, X. J., Shen, Y. F., 2006. Microcalorimetric investigation of the toxic action of Cr(IV) on the metabolism of *Tetrahymena thermophila* BF₅ during growth. *Environmental Toxicology and Pharmacology* 22:121-127.
240. Zheng, Z., He, P. - J., Shao, L. - M., Lee, D. - J., 2007. Phthalic acid esters in dissolved fractions of landfill leachates. *Water Res.* 41:4696-4702
241. Zhou, Q. H., Wu, Z. B., Cheng, S. P., He, F., Fu, G. P., 2005. Enzymatic activities in constructed wetlands and di-n-butyl phthalate (DBP) biodegradation. *Soil Biol Biochem* 37:1454–1459
242. Zilber, I. K., Rosenberg, E., Gutnick, D., 1980 Incorporation of ³²P and growth of *Pseudomonas* UP-2 on tetracosane. *Applied and Environmental Microbiology* 40:1086-1093.