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# GROWTH OF COLONIES AND HYPHAL ULTRASTRUCTURE OF FILAMENTOUS FUNGI GROWN ON DIBUTYL PHTHALATE AND DI (2-ETHYLHEXYL)PHTHALATE

# CRECIMIENTO DE COLONIAS Y ULTRAESTRUCTURA DE HIFAS DE HONGOS FILAMENTOSOS CULTIVADOS EN DIBUTIL FTALATO Y DI(2-ETILHEXIL)FTALATO

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**Abstract**

Phthalates are compounds that give flexibility to the plastics and are considered mutagens and teratogens. Mycelial growth rate, biomass production and hyphal diameter of the young and mature zones of colonies of *Fusarium oxysporum*, *Mortierella alpina*, *Pleurotus pulmonarius*, two strains of *Pleurotus ostreatus* (Po 37 and Po 83) and one strain of *Pleurotus florida* grown on glucose, di(2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate were studied. *F. oxysporum* had the highest mycelial growth rate on media containing DEHP (270 mm/d) than on medium added with glucose (90 mm/d). From all the fungi, *P. florida* produced the highest amount of biomass in medium containing glucose (280 mg/cm$^2$). *F. oxysporum* and *M. alpina* produced the highest amount of biomass in media containing DEHP (200 and 82 mg/cm$^2$, respectively). This research suggests that some of these fungi (e.g. *F. oxysporum* and *M. alpina*) are able to use these compounds as sole carbon and energy sources, and that the hyphal diameter of some strains was affected by these phthalates. However, further studies need to be carried out on physiology of fungi, on the effect of phthalates in the hypha ultrastructure and on the degradation of phthalates to increase our current understanding of the fungal biodegradation of these compounds.

*Keywords*: biodegradation, dibutyl phthalate, di (2-ethylhexyl) phthalate, filamentous fungi, mycelial growth.

**Resumen**

Los ftalatos son compuestos que proporcionan flexibilidad a los plásticos y son considerados mutágenos y teratógenos. En este trabajo se estudiaron la velocidad de crecimiento radial, la producción de biomasa y el diámetro de la hifa de las zonas joven y madura de colonias de *Fusarium oxysporum*, *Mortierella alpina*, *Pleurotus pulmonarius*, dos cepas de *Pleurotus ostreatus* (Po 37 and Po 83) y una cepa de *Pleurotus florida* crecidos sobre di (2-etilhexil) ftalato (DEHF) y dibutil ftalato. *F. oxysporum* presentó mayor velocidad de crecimiento radial en medio conteniendo DEHF (270 mm/d) que en medio conteniendo glucosa (90 mm/d). De todos los hongos estudiados, *P. florida* produjo la mayor cantidad de biomasa en el medio de cultivo adicionado con glucosa (280 mg/cm$^2$). *F. oxysporum* y *M. alpina* produjeron la mayor cantidad de biomasa en el medio conteniendo DEHF (200 y 82 mg/cm$^2$, respectivamente). Esta investigación sugiere que algunos de estos hongos (e.g. *F. oxysporum* y *M. alpina*) son capaces de utilizar estos compuestos como única fuente de carbono y energía, y que el diámetro de la hifa de estas cepas fue afectado por estos ftalatos. Sin embargo, es necesario llevar a cabo más estudios sobre la fisiología de estos hongos, sobre el efecto de estos compuestos en la ultraestructura de las hifas y sobre la degradación de ftalatos, para ampliar el conocimiento sobre la biodegradación de estos compuestos por hongos.

*Palabras clave*: biodegradación de ftalatos, crecimiento micelial, dibutil ftalato, di (2-etilhexil) ftalato, hongos filamentosos.

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# 1 Introduction

Plasticizers, including phthalate and phthalate esters, are widely used in the manufacture of polyvinyl chloride (PVC) to produce plasticized PVC as they greatly increase flexibility of the plastic enabling a wider range of products to be manufactured (Cartwright *et al*., 2000; Hauser and Calafat, 2005; Kim *et al*., 2007). As plasticizers form relatively weak hydrogen bonds within the fabric of PVC, over time phthalates can leach into the environment, especially if heated (Psillakis *et al*., 2004). These compounds have been found in food (Benson, 2009), products for personal consumption (Koniecki *et al*., 2011), dust, air from interior of apartments (Xu *et al*., 2010; Schripp *et al*., 2010), air from interior of vehicles (Geiss *et al*., 2009), soil and sediments. In addition, phthalates and their metabolites have also been found in breast milk (Fromme *et al*., 2011; Zhu *et al*., 2006; Hines *et al*., 2009; Main *et al*., 2006). The Phthalate Information Center maintains that the risk of phthalates to human reproductive and developmental health is minimal or negligible, however, independent studies have shown phthalates to be endocrine disruptors that may affect development in animals (Matsumoto *et al*., 2008). The most widely used phthalates are the di (2-ethylhexyl) phthalate (DEHP), followed by dibutyl phthalate (DBP). The degradation of phthalates have been mainly studied by using bacteria, however, it has also been reported that fungi are able to degrade these compounds (Rivera-Cruz *et al*., 2002; Luo *et al*., 2012; Hwang *et al*., 2008; Hwang *et al*., 2012). On the other hand, Sánchez *et al*. (2004) reported that the content of cytoplasmic material and thickness of the cell wall (= ultrastructure) are important to determine the maturity of the hypha and the mycelial growth of a fungal colony. In this study, we report the radial growth rate, biomass production, and diameter of the hypha of young zone (YZ) and mature zone (MZ) of colonies of *Fusarium oxysporum*, *Mortierella alpina*, *Pleurotus pulmonarius*, *Pleurotus florida* and P. ostreatus grown on DEHP and DBP.

# 2 Materials and methods

## *2.1 Organisms*

Six strains were used in this study; *Fusarium oxysporum* Schltdl. isolated from soil of the suburbs in central Manchester (Manchester, UK), *Mortierella alpina* Peyrone. isolated from a park in central Manchester (Manchester, UK), *Pleurotus pulmonarius* (Fr.) Quél. from the Chinese University of Hong Kong Collection (Shatin, Hong Kong), two strains of *Pleurotus ostreatus* (Jacq.) P. Kumm. from the ATCC (American Type Culture Collection) (Manassas, Virginia, USA), ATCC-38537 (Po37) and ATCC-32783 (Po83) and one strain of *Pleurotus florida* nomen nudum from the Universidad Autónoma de Tlaxcala collection (Tlaxcala, Mexico). Stock cultures were grown on malt extract agar (MEA) in the dark at 25ºC and then stored at 4ºC. Cultures were transferred to fresh culture media periodically. In all the studies, mycelial plugs (4 mm diam) from the periphery of colonies grown on MAE were used as inoculum.

## *2.2 Culture media and culture conditions*

Six different culture media were prepared; C1) 50 ml of mineral salt medium (MM), C2) 50 ml of MM + 500 mg of DEHP/l, C3) 50 ml of MM + 1000 mg of DEHP/l, C4) 50 ml of MM + 500 mg of DBP/l, C5) 50 ml MM + 1000 mg of DBP/l, and C6) 50 ml of MM + 10 g/l of glucose. The MM used contained (g/l): $KH_2PO_4$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; $(NH_4)_2SO_4$, 1.0; $CaH_4(PO_4){\cdot}H_2O$, 0.3; $FeSO_4{\cdot}7H_2O$, 0.2; $ZnSO_4{\cdot}7H_2O$, 0.2; $MnSO_4{\cdot}H_2O$, 0.2 and bacteriological agar, 20. The pH was adjusted to 6.0 using 0.1 and 1.0 M of NaOH. DEHP (SIGMA-ALDRICH D201154, 99%) and DBP (SIGMA-ALDRICH 524980, 99%) were added by separated to the sterile MM and then the culture media were sonicated for approximately 3 minutes using an ultrasonic processor (GEX 130) until the DEHP and DBP had fully dispersed.

## *2.3 Radial growth rate and biomass*

The radial growth rate ($u_r$) was calculated as the slope of the radius versus time plots, analyzed by lineal regression. The radius was measured daily from the $2^{nd}$ to the $7^{th}$ d of incubation (Sánchez and Viniegra-Gonzaléz, 1996). The mycelial biomass was evaluated in 7 seven days old colonies by placing the mycelium separated from the culture medium using a hot-water bath in a pre-weighed, watch glass. This was weighed, and then oven-dried at 60 °C for 24 h, then weighed again (AOAC, 1990).

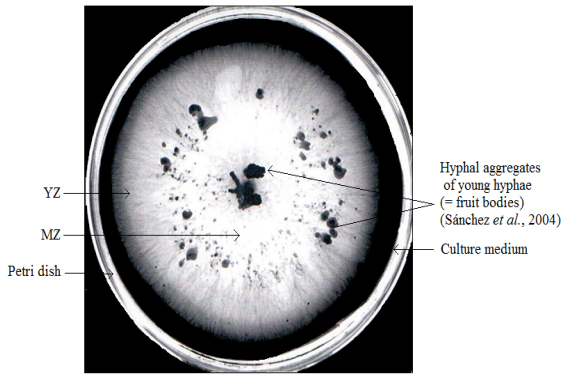


Fig. 1. Differentiation of the mature and young zones of growth of a colony of *P. pulmonarius* grown on potato dextrose agar and stained with Feulgen reagent.

### 2.4 *Differentiation zone of grown*

Feulgen reagent was added to seven day-old colonies grown on the different culture media at 25°C in Petri dishes, and incubated for 20 min at room temperature (Sánchez *et al*., 2004). Feulgen reagent contains HCl and fuchsine, the HCl breakdown the cell wall and then fushine stains the cytoplasmic material. Thin cell wall is stained quicker than thick cell wall. The dye was removed and the Petri dishes were rinsed with distilled water and dried at room temperature. The unstained hyphae correspond to the MZ of the colony or mature hyphae. The stained hyphae from the periphery of the colony correspond to the YZ of the colony or young hyphae. The center of the colony that corresponds to the inoculum (oldest hyphae) is also stained. The hyphae undergo lysis after 8 or 10 d of fungal growth, releasing glucans and other compounds that are used in the growth of new hyphae. This event leads to the formation of young hyphae in the MZ of the colony (Fig. 1) (Sánchez *et al*., 2004).

### 2.5 *Measurement of the diameter of the hypha*

In stained colonies, pieces from mycelium of 2 $cm^2$ were cut from the YZ and from the MZ of the colonies and were placed in a glass, and then the diameter of the hypha (Fig. 2) was measured using a computerized image analysis system (Image-Pro Plus Version 4.0 for windows, 1999 Media Cybernetics, L.P.). The results are reported as a relation diameter of the hyphae from the YZ between diameter of the hyphae from the MZ (YZ/MZ). The diameter was measured in $\mu$m.

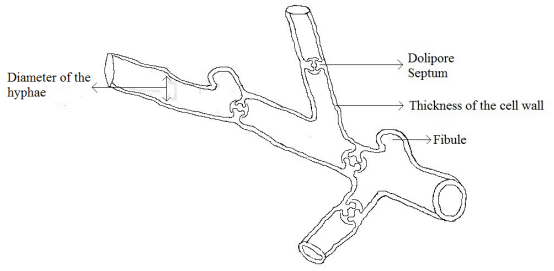


Fig. 2. Schematic representation of the hyphal structure.

## 3 Results and discussion

The strain of *F. oxysporum* showed the highest $u_r$ in both phthalates. It also showed greater $u_r$ in those media containing DEHP than in those media containing glucose (optimum medium). It has been reported that cutinases are enzyme able to degrade phthalates (Kim *et al*., 2007). Cartwright *et al*., (2000) reported that *F. oxysporum* was able to produce extracellular cutinases. The highest $u_r$ of *P. pulmonarius* was observed in media containing 1000 mg of DEHP/l. *M. alpina* had the highest and the lowest $u_r$ on 500 mg/l of DEHP and MM, respectively (Table 1). The strains grew better on media containing DEHP than on those media containing DBP. Some fungi were able to grow on MM, this could be due to the use of certain amount of nutrients that unavoidably remain in the inoculum and/or the use of nutrients produced by cellular lysis of the hypha from the inoculum. In general, the strains showed the highest biomass production in the medium containing glucose, followed by the medium containing DEHP (Table 2). The strains produced similar amount of biomass in media containing 500 and 1000 mg of DBP/l. *P. florida* produced the highest biomass production followed by Po83 in medium added with glucose. On the other hand, it has been reported a method that can differentiate the MZ and YZ of a fungal colony and it was found that the differentiation of both zones of the colony was due to the hyphal wall thickness and to the citoplasmic material content. It was observed that the wall from hyphae of the MZ of *P. pulmonarius* grown on MEA was twice thicker and had lower glycogen content than those hyphae from the YZ of the colony (Sánchez *et al*., 2004). In this research, the MZ and YZ of the colonies grown on media containing phthalate were also differentiated.

Table 1. Radial growth rate (mm/d) of filamentous fungi grown for 7 d at 25°C on different culture media.

| Strain | Culture media | | | | | |
|---|---|---|---|---|---|---|
| | MM | Glucose | MM+500 mg of DBP /l | MM+1000 mg of DBP /l | MM+500 mg of DEHP /l | MM+1000 mg of DEHP /l |
| | $10^{-3}$ | | | | | |
| *P. pulmonarius* | $90^{d}$ (4) | $180^{b}$ (12) | $70^{e}$ (2) | $50^{f}$ (2) | $120^{d}$ (18) | $190^{a}$ (14) |
| *P. ostreatus* 37 | $120^{d}$ (14) | $250^{a}$ (18) | $60^{e}$ (1) | $70^{d}$ (3) | $220^{b}$ (14) | $230^{b}$ (12) |
| *P. florida* | $100^{b}$ (4) | $240^{a}$ (20) | $50^{e}$ (0.6) | $40^{f}$ (2) | $90^{d}$ (3) | $80^{d}$ (5) |
| *P. ostreatus* 83 | $100^{d}$ (10) | $290^{a}$ (17) | ng | ng | $230^{b}$ (6) | $60^{d}$ (2) |
| *M. alpina* | $50^{e}$ (1) | $170^{b}$ (10) | $150^{d}$ (11) | $70^{d}$ (2) | $200^{a}$ (18) | $170^{b}$ (10) |
| *F. oxysporum* | $180^{b}$ (6) | $90^{d}$ (2) | $170^{d}$ (6) | $160^{d}$ (7) | $270^{a}$ (10) | $260^{a}$ (14) |

Means with the same letter within a row are not significantly different. Data were evaluated ANOVA and Tukey test. $P < 0.01$). Numbers in parenthesis correspond to SD of three separate experiments.
ng: no growth.

Table 2. Biomass (mg/cm$^2$) of filamentous fungi grown for 7 d at 25 °C on different culture media.

| Strain | Culture media | | | | | |
|---|---|---|---|---|---|---|
| | MM | Glucose | MM+500 mg of DBP /l | MM+1000 mg of DBP /l | MM+500 mg of DEHP /l | MM+1000 mg of DEHP /l |
| | $10^{-3}$ | | | | | |
| *P. pulmonarius* | $20^{f}$ (0.02) | $122^{a}$ (3.0) | $46^{d}$ (0.03) | $35^{e}$ (0.02) | $80^{d}$ (0.4) | $110^{b}$ (0.3) |
| *P. ostreatus* 37 | $50^{d}$ (0.01) | $120^{a}$ (1.0) | $30^{e}$ (0.01) | $36^{e}$ (0.03) | $105^{c}$ (1) | $110^{b}$ (0.9) |
| *P. florida* | $45^{e}$ (0.07) | $225^{a}$ (3.0) | $60^{d}$ (0.02) | $46^{e}$ (0.03) | $105^{b}$ (3) | $93^{c}$ (0.05) |
| *P. ostreatus* 83 | $45^{c}$ (0.09) | $200^{a}$ (4.0) | ng | ng | $160^{d}$ (2) | $40^{d}$ (0.06) |
| *M. alpina* | $13^{e}$ (0.01) | $70^{b}$ (0.9) | $65^{c}$ (0.7) | $30^{d}$ (0.03) | $82^{a}$ (2) | $70^{b}$ (0.9) |
| *F. oxysporum* | $73^{d}$ (0.01) | $110^{d}$ (3) | $160^{b}$ (0.09) | $120^{d}$ (0.03) | $200^{a}$ (0.01) | $190^{a}$ (0.07) |

Means with the same letter within a row are not significantly different. Data were evaluated ANOVA and Tukey test. $P < 0.01$). Numbers in parenthesis correspond to SD of three separate experiments.
ng: no growth

Table 3. Relation diameter of YZ/MZ of filamentous fungi grown for 7 d at 25 °C on different culture media.

| Strain | Culture media | | | | | |
|---|---|---|---|---|---|---|
| | MM | Glucose | MM+500 mg of DBP /l | MM+1000 mg of DBP /l | MM+500 mg of DEHP /l | MM+1000 mg of DEHP /l |
| | YZ/ MZ | YZ/ MZ | YZ/ MZ | YZ/ MZ | YZ/ MZ | YZ/ MZ |
| *P. pulmonarius* | $0.80^{d}$ | $1.20^{a}$ | $1.11^{c}$ | $1.09^{c}$ | $0.81^{d}$ | $1.16^{b}$ |
| *P. ostreatus* 37 | $0.88^{c}$ | $0.78^{d}$ | $1.12^{a}$ | $1.14^{a}$ | $0.93^{b}$ | $0.68^{e}$ |
| *P. florida* | $0.94^{d}$ | $0.81^{e}$ | $1.12^{b}$ | $0.97^{c}$ | $0.90^{d}$ | $1.21^{a}$ |
| *P. ostreatus* 83 | $0.99^{b}$ | $0.98^{b}$ | ng | ng | $1.17^{a}$ | $0.99^{b}$ |
| *M. alpina* | $0.67^{c}$ | $0.57^{d}$ | $0.79^{b}$ | $1.58^{a}$ | $0.79^{b}$ | $1.58^{a}$ |
| *F. oxysporum* | $1.07^{a}$ | $0.76^{c}$ | $0.80^{d}$ | $1.06^{a}$ | $1.4^{b}$ | $1.68^{a}$ |

ng: no growth

On the basis of the studies reported before, this suggests that the hyphae from the MZ presented a twice thicker cell wall and lower amount of glycogen than those hyphae from the YZ of the colony. This suggests that the fungi are using these phthalates to grow (Table 3). The differentiation of both zones of growth was not observed in the strain Po83 grown on media containing DBP. On the basis of the studies reported by Sánchez *et al*. (2004), it suggests that this strain presents a similar cell wall thickness and glycogen content in both zones of growth of the colony. In general, it was observed an effect of the phthalates in the diameter of the hyphae, since the values of the relation of the diameter of the hyphae from the YZ/MZ of the colonies grown on media added with phthalates and of the colonies grown on medium added with glucose was significantly different. In *Fusarium oxysporum*, the value of the relation of the diameter of the hyphae from the YZ/MZ of colonies grown in media containing 1000 mg/l of DEHP and 1000 mg/l of DBP was significantly higher than that value of the relation of the diameter of the hyphae from the YZ/MZ of colonies grown in media added with glucose (Table 3). The strain Po83 did not grow in the medium containing DBP. The atypical behavior of this strain might be due to the specific enzymes that each strain presents and to the different pathway for phthalates degradation that might be used. This research suggest that some of these fungi are able to degrade and use phthalates as only carbon and energy sources and that in some fungi the diameter of the hyphae was affected by these phthalates, However, further studies need to be carried out about the physiology of fungi, effect of phthalates on the hyphal ultrastructure and degradation of phthalates to increase our current understanding of fungal biodegradation of these compounds. The knowledge of these aspects is of crucial importance to select strains able to degrade DBP and DEHP that can be used in bioremediation of phthalate-contaminated environments.

## References

AOAC. (1990). *Association of Official Analytical Chemists*, 15th edn. Arlington, Va.

Benson, R. (2009). Hazard to the developing male reproductive system from cumulative exposure to phthalate esters-dibutyl phthalate, diisobutyl phthalate, butyl benzyl phthalate, diethylhexyl phthalate, dipentyl phthalate, and diisononyl phthalate. *Regulatory Toxicology and Pharmacology 53*, 90-101.

Cartwright, C.D., Owen, S.A., Thompson, I.P. y Richard, G. (2000). Biodegradation of diethyl phthalate in soil by a novel pathway. *FEMS Microbiology Letters 186*, 27-34.

Fromme , L., Gruber, E., Seckin, U., Raab, S., Zimmermann, M., Kiranoglu, M., Schlummer, U., Schwegler, S., Smolic, W. y Völkel. (2011). Phthalates and their metabolites in breast milk - Results from the bavarian monitoring of breast milk (BAMBI). *Environment International 37*, 715-722.

Geiss, O., Tirendi, S., Barrero-Moreno, J. y Kotzias, D. (2009). Investigation of volatile organic compounds and phthalates present in the cabin air of used private cars. *Environment International 35*, 1188-1195.

Hauser, R. y Calafat A.M. (2005). Phthalates and human health. Occupational and Environmental Medicine 62, 806-818.

Hines, E.P., Calafat, A.M., Silva, M.J., Mendola, P. y Fenton, S.E. (2009). Concentrations of phthalates metabolites in milk, urine, saliva, and serum of lactating North Carolina women. *Environmental Health Perspectives 117*, 86-92.

Hwang, S.S., Choi, H.T. y Song, H.G. (2008). Biodegradation of endocrine-disrupting phthalates by *Pleurotus ostreatus*. *Journal Microbiology and Biotechnology 18*, 767-772.

Hwang, S.S., Kim, H.Y., Ja, K.O. y Song, H.G. (2012). Changes in the activities of enzymes involved in the degradation of butylbenzyl phthalate by *Pleurotus ostreatus*. *Journal Microbiology and Biotechnology 22*, 239-243.

Kim, Y.H., Seo, H.S., Min, J., Kim, Y.C., Ban, Y.H., Han, K.Y., Park, K.D., Bea, M.B. y Lee, J. (2007). Enhanced degradation and toxicity reduction of dihexyl phthalate by *Fusarium oxysporum* f. sp. Pisi cutinase. *Journal Applied Microbiology 102*, 221-228.

Koniecki, D. y Wang, R. (2011). Phthalates in cosmetic and care product: concentrations and posible dermar exposure. *Environmental Research 111*, 329-336.

Luo, Z.H., Pang, K.L., Wu, Y.R., Gu, J.D., Chow, R.K. y Vrijmoed, L.L. (2012). Degradation

of phthalates esters by *Fusarium* sp. DMT-5-3 and *Trichosporon* sp. DMI-5-1 isolated from mangrove sediments. *Progress in Molecular and Subcellular Biology 53*, 299-328.

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. y Skakkebæk, N.E. (2006). Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environmental Health Perspectives 114*, 270-276.

Matsumoto, M., Hirata-Koizumo, M. y Ema M. (2008). Potential adverse effects of phthalic acid esters on human health: a review of recent studies on reproduction. *Regulatory Toxicity Pharmaceutical 50*, 37-49.

Psillakis, E., Mantzavinos, D. y Kalogerakis, N. (2004). Monitoring the sonochemical degradation of phthalate esters in water using solid-phase microextraction. *Chemosphere 54*, 849-857.

Rivera-Cruz M.C., Ferrera-Cerrato R., Volke-Haller, V., Fernández-Linares, L. y Rodríguez-Vázquez, R. (2002). Poblaciones microbianas en perfiles de suelos afectados por hidrocarburos del petróleo en el Estado de Tabasco, México. *Agrociencia 36*, 149-159.

Sánchez, C., Téllez-Téllez, M., Díaz-Godínez, G. y Moore, D. (2004). Simple staining detects ultrastructural and biochemical differentiation of vegetative hyphae and fruit body initials in colonies of *Pleurotus pulmonarius*. *Letters Applied Microbiology 38*, 483-487.

Sánchez, C., y Viniegra-Gonzaléz, G. (1996). Detection of highly productive strains of *Pleurotus ostreatus* by their tolerance to 2-deoxy-D-glucose in starch-based media. *Mycological Research 100*, 455-461.

Schripp, T., Fauck, C. y Salthammer, T. (2010). Chamber studies on mass-transfer of di(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DnBP) from emission sources into hause dust. *Atmospheric Environment 44*, 2840-2845.

Xu, Y., Humbal, E.A.C. y Little, J.C. (2010). Predicting residential exposure to phthalate plasticizer emitted from vinyl flooring: sensitivity, uncertainty, and implications for biomonitoring. *Environmental Health Perspectives 118*, 253-558.

Zhu, J., Phillips, S.P., Feng, Y.L. y Yang, X. (2006). Phthalates esters in human milk: concentration variation over a six-month pospartum time. *Environmental Science and Technology 40*, 5276-5281.