TITLE: Comparison of solid-phase and eluate assays to gauge the ecotoxicological risk of organic wastes on soil organisms

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ABSTRACT

Development of methodologies to assess the safety of reusing polluted organic wastes in soil is a priority in Europe. In this study, and coupled with chemical analysis, seven organic wastes were subjected to different aquatic and soil bioassays. Tests were carried out with solid-phase waste and three different waste eluates (water, methanol, and dichloromethane).

Solid-phase assays were indicated as the most suitable for waste testing in terms of relevance for real situations, but also because toxicity in eluates was generally not representative of the chronic effects in solid-phase.

No general correlations were found between toxicity and waste pollutant burden, neither in solid-phase nor in eluate assays, showing the inability of chemical methods to predict the ecotoxicological risks of wastes. On the contrary, several physico-chemical parameters reflecting the degree of low organic matter stability in wastes were the main contributors to the acute toxicity seen in collembolans and daphnids.

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SENTENCE CAPSULE: Comparison of solid-phase and eluate bioassays for organic waste testing.

KEYWORDS: Organic wastes ecotoxicity; Solid-phase tests; Eluate tests.

1. INTRODUCTION

There is increasing interest in the development of bioassays to evaluate the suitability of polluted organic wastes for safe application to soils excluding any ecotoxicological risk. The complex nature of such wastes, especially in the case of sewage sludge, containing a huge number of potentially noxious chemicals (Thornton et al. 2001), and the limitations of chemical methods to assess their risk to soils (Crouau et al. 2002) favours using a bioassay approach.

A wide variety of literature dealing with potential effects on crops of polluted organic wastes is available, mainly centred on sewage sludge, while less is known about effects on soil fauna, a key group in soil agroecosystems (Giller et al. 1997, Neher 1999). No harmful effects on soil fauna have been found in field studies using agronomic dosages (Cole et al. 2001, Kielhorn et al. 1999, Krogh et al. 1997, Petersen et al. 2003), although some laboratory studies have indicated risk for soil fauna if such wastes are applied to soils (Andrés and Domene 2005, Krogh et al. 1997, Krogh and Pedersen 1997). This scarcity of studies shows how incomplete the knowledge on this subject is and is a sign

of the current need for the selection of bioassays to assess the ecotoxicological risk of wastes to soils.

Among the laboratory studies centered on organic waste ecotoxicity, most have been carried out using waste eluates or leachates and aquatic ecotoxicity tests. However, others have focused on the suitability of solid-phase bioassays for organic wastes using terrestrial organisms. The latter approach is the most relevant as it provides results closer to those expected in field conditions. However, solid-phase assays have several drawbacks associated to the organic matter matrix. Organic matter may mask toxicity through its nutritive effect on soil organisms, but also may modify physicochemical properties and the water holding capacity of the soil-waste mixtures at increasing concentrations, therefore affecting to a certain extent the performance of the test organism.

Assays on the aqueous eluate of waste is the most commonly employed method for waste ecotoxicity assessment (Vaajasaari 2005). For organic wastes, this approach has been taken using microorganisms, plants, and daphnids as test organisms. However, to date, correlation of results with those obtained from solid-phase tests has not been reported to our knowledge, indeed the extrapolation of results from aqueous eluates and aquatic bioassays to soil organisms has been criticized for its low ecological relevance (Alexander et al. 2003, McMillen et al. 2003). Despite these criticisms, the use of eluates may compensate for some of the main limitations of the solid-phase tests, as long as results of these eluate assays correlate with those of the solid-phase assays. Furthermore, combined testing of different eluate solvents (water and organic solvents) may give information about the pollutant fraction mainly contributing to the harmful effects of wastes.

The aims of this work are (a) to compare sensitivities of terrestrial and aquatic assays for the ecotoxicity assessment of organic wastes; (b) to examine the representativeness of different waste eluates for the estimation of solid-phase toxicity; and (c) to detect the main contributors to the organic waste toxicity by correlating the biological response with waste composition both in solid-phase and eluate assays. For this purpose, organic waste in solid-phase and its corresponding three eluates (water, methanol and dichloromethane), obtained with a method incurring low experimental and economic costs, were compared by chemical methods and bioassays (survival and reproduction of the soil collembolan *Folsomia candida*, luminescence of the marine bacteria *Vibrio fischeri*, and mobility of the freshwater copepod *Daphnia magna*).

MATERIALS AND METHODS

2.1. Sample origin and preparation

Six different sewage sludges and one thermally dried pig slurry were selected. Given their contrasting origins and treatments, these samples are representative of a broad range of organic wastes generated in Europe (Table 1).

Each waste was dried at 60ºC for 48-72 hours depending on its initial moisture, and then ground and sieved (<2 mm), in order to ensure the homogeneity and accuracy of the lowest test concentrations and for the preparation of eluates.

2.2. Characterization of wastes and eluates

The most relevant physicochemical properties of the original wastes are recorded in Table 2, and were determined on the same sample allotment used for bioassays. Dry matter and ammonium were determined from fresh samples, while the remaining parameters were measured from the dry and ground samples. Dry matter, water holding

capacity, pH, electric conductivity, total nitrogen, and organic matter were measured according to EN 12880, ISO 11267, EN 13037, EN 13038, EN 13342 and EN 12879 (CEN 1999a, 1999b, 2000a, 2000b, 2000c). Non-hydrolysable (stable) organic matter and non-hydrolysable nitrogen were measured as the percentage of organic matter and nitrogen remaining in the sample residue after acid hydrolysis, as described in Rovira and Vallejo (2002). Hydrolysable nitrogen was the difference between total nitrogen and non-hydrolysable nitrogen. Ammonium was measured by distillation of the fresh sample.

Three different solvents (water, methanol and dichloromethane) were used to prepare different waste eluates, in order to isolate three pollutant fractions, differing in their solubility. Eluates were prepared with deionised water, methanol (99.5% purity), and dichloromethane (99.8% purity) (SDS Chemicals, Peypin, France). Aqueous elution allows dissolution of hydrophylic (polar) substances and methanol elutes amphyphylic substances (semipolar) while dichlorometane solubilizes hydrophobic (non-polar) substances. The procedures used to prepare the eluates in each bioassay are are detailed in later sections.

Pollutant burden of wastes and eluates used in *F. candida* and Microtox assays are recorded in Table 3. No chemical data were available from the aqueous eluates specifically prepared for the *D. magna* assay and those for the Microtox assays. However, we assumed that relative differences in chemical burden between wastes were equivalent to that of the aqueous eluates prepared used in assays of *F. candida*, for which direct measures were available. Heavy metals in wastes were determined by Applus Agroambiental Inc., while heavy metals in eluates were determined by the Chemical Analysis Service of the Autonomous University of Barcelona. Organic pollutants were determined by the Sarrià Chemical Institute of Barcelona. Cd, Cr, Cu,

Hg, Ni, and Pb were determined by ICP-MS according to ISO 11885 (ISO 1996a). Polychlorinated dibenzodioxins/dibenzofuranes (PCDD/F) were measured with HRGC-HRMS, polychlorinated biphenyls (PCB) with HRGC-ECD, di(2-ethylhexyl)phthalate (DEHP) and NPE with HRGC-MS. Polycyclic aromatic hydrocarbons (PAH) and linear alkylbenzene sulphonates (LAS) were measured with HPLC with fluorescence and UV detectors respectively. NPE include nonylphenol and nonylphenol ethoxylates with 1 or 2 ethoxy groups. PAH were the sum of acenapthene, phenanthrene, fluorene, flouranthene, pyrene, benzo(b+j+k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, and indeno(1, 2, 3-c, d)pyrene. PCB was the sum of the polychlorinated biphenyl congener number 28, 52, 101, 118, 138, 153 and 180.

2.3. *Folsomia candida* reproduction assay

F. candida is a soil collembolan commonly used in soil ecotoxicity tests. The assay was performed in artificial soil according to ISO 11267 (1999). Effects on survival and reproduction at increasing concentrations of waste or eluate were measured after 28 days of exposure, the time needed to obtain a first generation from the parent individuals.

Eluates were prepared by sonication of the dried and ground wastes in contact with the extraction solvent. More precisely, 200 g of dried and ground waste were added to 500 ml of solvent in an Erlenmeyer flask and placed in an ultrasound extraction system for 90 minutes at room temperature. Methanol and dichloromethane eluates were then vacuum-filtered with cellulose filter (0.45-µm poresize), and concentrated in a rotavapour. Aqueous eluates were centrifuged, decanted, vacuum-filtered and concentrated by lyophilization. Final concentrations of each eluate were adjusted to

2500 g L^{-1} by addition of pure solvent. Solid matter in eluates was low (5.6, 4.2, and 3% mean values in water, methanol, and dichloromethane eluates, respectively).

For the solid-phase assays, toxicity results of *F. candida* were obtained from Domene et al. (2007). In solid-phase assays, twelve test concentrations were prepared by mixing dry waste with artificial soil at 0, 1, 2, 4, 7.9, 15.8, 31.6, 63.1, 125.9, 251.2, 501.2 and 1000 g Kg^{-1}. In the eluate assays, the suitable volume of extract was added to the soil to obtain the same test concentrations used in the solid-phase assays, calculated according to the total waste weight initially used to prepare the extracts. In order to ensure the similitude of treatments in the different concentrations, this suitable volume of extract was raised to a constant volume by the addition of pure solvent. The same volume of pure solvent was also added to controls. Then, methanol and dichloromethane soil-eluate mixtures were left to evaporate completely for 48 hours in a fume hood at room temperature. For soil-aqueous eluate mixtures, drying was carried out in a stove at 60ºC for 48 hours. Once dry, soil-eluate mixtures were homogenized and deionised water was added to obtain a water content around 55% (w/dw).

Five replicates per concentration were prepared, consisting of 125 ml polyethylene sealed containers with 30 g of wet mixture. In each replicate, 10 individuals 10 to 12 days old were added together with 3 mg of granulated dry yeast. Containers were kept in the dark at 21±1ºC for 28 days, and were aerated twice a week. Yeast was also added the 14th day of the test.

At the end of this period, containers were flooded with water to float the adults and juveniles on the surface. A dark dye was used to facilitate the counting of the individuals, and a picture was taken to assess effects on several endpoints using the image treatment software ImageTool 3.0. From pictures, the number of adults and juveniles per replicate was determined, which were distinguishable by their size. From

these data, relative survival (100*number of adults in the replicate/mean number of adults in controls) and relative reproduction (100*number of juveniles in the replicate/mean number of juveniles in controls) were calculated.

The survival and reproduction of the individuals in the eluate assays were compared with the controls in solid-phase assays using one-way ANOVA in order to detect any adverse effects of the solvents.

2.4. Microtox acute toxicity assay

This assay was selected due to its common use in aquatic ecotoxicity and to infer potential adverse effects on soil organisms from soil eluates. Microtox acute toxicity test was carried out by the Sarrià Chemical Institute of Barcelona, according to ISO 11348 (1998), and using a Microtox Model 500 Analyzer (Azur Environmental Inc.). The assay is based on the luminescence inhibition of the strain NRRL B-11177 of the marine bacteria *Vibrio fischeri*. Luminescence is a by-product of its cellular respiration, so any adverse effect on its metabolism is reflected by a loss in bioluminescence. Luminescence inhibition of cultures at 15ºC after 15 minutes of exposure was used as the endpoint.

For the Microtox acute toxicity assay, based on seawater bacteria, the eluates have to be applied to an aqueous environment. For this, 2 ml of each eluate were dissolved in Microtox saline solution (2 % NaCl) as indicated in ISO 11348 (1998), to a final volume of 50 ml. For methanol and dichloromethane, previous to this dissolution, 2 ml aliquots were subjected to a continuous flow of nitrogen until complete evaporation of solvent. Then, 50 ml of saline solution was added to the solid remnant and the mixture was sonicated for 1 hour. Finally, the resultant solution was vacuum-filtered in a 0.45 μm pore size filter. Hence, we obtained a final eluate concentration of 100 g L^{-1} for the

196 aqueous, methanol, and dichloromethane eluate assays. For this assay, an additional
197 eluate was prepared, herein called solid-phase eluate, consisting of an aqueous eluate
198 from wastes. 5 g of dry and ground waste were mixed with 50 ml of saline solution.
199 Then, the mixture was sonicated for 1 hour, and finally vacuum-filtered in a 0.45 μm
200 filter. Hence, a final eluate concentration of 100 g L^{-1} was also obtained and used for
201 preparation of dilutions.
202 Assays were performed by duplicate with the eluates already described. Four test
203 concentrations (0, 5.6, 11.25, 22.5, and 45% of eluate) were prepared from dilutions of
204 the primary eluates. When these concentrations did not inhibit luminescence, dilutions
205 of the primary eluates (1:5, 1:10, or 1:20) were used.
206
207
208 **2.5. *Daphnia magna* acute toxicity assay**
209 *D. magna* is a freshwater copepod widely used for aquatic ecotoxicological purposes
210 and also for soil ecotoxicity using soil eluates. Assays were performed in Environmental
211 Toxicology Laboratory of the Technical University of Catalonia according to ISO 6341
212 (1996b). The endpoint for toxicity assessment is inhibition of the individuals' mobility
213 after 48 hours of exposure.
214 For *Daphnia magna* acute toxicity assays, eluates were obtained as indicated in EN
215 12457-2 (CEN 2002), a procedure used to assess the risk of pollutants leaching from
216 granular wastes or sludge. Extraction was performed by adding 900 ml of deionised
217 water to 90 g of dry and ground waste, and shaking for 24 hours at room temperature.
218 After 15 minutes of sedimentation, liquid was decanted and the eluate was centrifuged
219 for 30 minutes at 3500 g, decanted again, and vacuum-filtered in a 0.45 μm pore size
220 filter. Only solid-phase aqueous eluates were prepared for this assay, since according to

this method, extraction has to be carried out with deionised water, and given the practical limitations of the high volume of waste and extraction solvent that would be required for the preparation of methanol and dichloromethane eluates.

From the solid-phase aqueous eluate, a dilution range was prepared to provide eight test concentrations (0, 1.5, 3, 6, 12.5, 25, 50, and 100% of eluate). One replicate per concentration was prepared, consisting of a glass flask with 20 individuals. Individuals were exposed to the test concentrations at 20ºC±2ºC in a constant light-dark photoperiod (16:8). After 48 hours, the number of immobilized individuals was determined.

2.6. Data treatment

In the *F. candida* assay, LC50 and EC50 values, together with 95% confidence intervals, were calculated by suitable regression models (exponential, Gompertz, hormesis, linear and logistic) using the Statistica 6.0 software package (StatSoft Inc.). The choice of the model was based on best fit to data according to Stephenson et al. (2000).

In the Microtox assay, luminescence inhibition values (EC50) and 95% confidence intervals were determined by means of the MicrotoxOmni software (Azur Environmental Inc.).

In the *D. magna* assay, the individual's mobility EC50 and 95% confidence intervals were determined by means of probit analysis using the statistical software package Minitab 13.2 (Minitab Inc. 2000).

In order to compare toxicity results of the different assays, Pearson correlations of their log-transformed toxicity values (LC50, EC50) were determined, using the statistical

245 package SPSS 13.0. Furthermore, within each bioassay, correlations of toxicity in the
246 solid-phase with that in eluates were sought by the same method.
247 Pearson correlations were also used to reveal which waste parameters were primarily
248 responsible for the observed toxicity in each bioassay. More precisely, significant
249 correlations of toxicity with the log-transformed values of individual pollutant
250 concentration, the sum of concentrations of pollutant groups, and physicochemical
251 parameters were sought. The assessed pollutant groups were heavy metals, organic
252 pollutants, persistent organics (PAH, PCB and PCDD/F), non-persistent (DEHP, LAS
253 and NPE), and total pollutant burden. When a concentration value was below the
254 detection limit, zero was used in the correlations.
255
256 **3. RESULTS**
257
258 **3.1. Sample and eluate characterization**
259 The studied wastes showed varied physicochemical properties and pollutant burden
260 (Table 2 and 3). Sludge compost showed higher organic matter stability and lower total
261 nitrogen content, with hydrolysable nitrogen and ammonium. Furthermore, composting
262 reduced concentrations of non-persistent organic pollutants (DEHP, NPE and LAS).
263 Thermal drying reduced the $N\text{-}NH_4$ levels and increased its electrical conductivity with
264 respect to dewatered sludge. No important reduction in the pollutant levels was
265 observed, except for DEHP, which showed a slightly lower concentration in the
266 thermally dried than in the dewatered sludges. In comparison to the other wastes,
267 thermally dried pig slurry presented low moisture content, extremely high electric
268 conductivity, was highly hydrolysable and had a high total nitrogen content and a low
269 organic matter stability.

Comparison between the concentration of a particular pollutant in the three eluates and the measured "total" concentration in the solid-phase (Table 3), gives an estimation of the extraction efficiencies of each solvent. Heavy metals were mainly in non-extractable form, as mean extraction efficiency for them was 0 to 4% for any solvent, except for Cd (43%) and Hg (15%) in the aqueous eluates. LAS, PCB, and PCDD/Fs were not detectable in eluates. NPE and PAH were not recovered in aqueous eluates, but were present in the methanol (48 and 37% respectively) and dichloromethane (37 and 24% respectively) eluates. DEHP showed high relative concentrations in methanol (80%), but was particularly high in dichloromethane eluates (246%), in which higher concentrations were found in eluates than in the solid-phase in some of the wastes. This unexpected result could be attributed to a higher extraction efficiency of dichloromethane with respect to the method used for "total" DEHP determination, or to an accidental loss of solvent in the dichloromethane eluates previous to DEHP determination, given the high volatility of dichloromethane. Given that DEHP concentrations in methanol and dichloromethane eluates were correlated with their concentrations in solid-phase, we assumed this result did not invalidate data for correlations with bioassays.

As a general trend, individual heavy metal concentrations in solid-phase were uncorrelated with their concentrations in eluates. On the contrary, solid-phase organic pollutant concentrations were generally correlated with their concentrations in methanol eluates ($p<0.05$ for DEHP, LAS, NPE, and PAH, respectively), and in dichloromethane eluates ($p<0.05$ for DEHP, NPE, and PAH, respectively), but not with aqueous eluates as no organic pollutants were detectable.

3.2. Bioassays comparison

Results from the battery of tests generally concord that sludge composts are the least toxic of the wastes studied, both when solid-phase or eluates are tested (Table 4, Figure 1). The exception was the Microtox assay using aqueous and dichloromethane eluates, which failed to identify composts as the least polluted wastes. On the contrary, there was no consistent pattern for the most toxic wastes, which depended on the bioassay and the pollutant fraction. This is why no correlations between bioassays could be found, with the exception of *F. candida* mortality in solid-phase tests which correlated highly with inhibition of mobility in *D. magna* ($r = 0.922$, $p=0.009$).

Different relative sensitivities of the various bioassays appeared when the different pollutant fractions were compared for their mean toxicity results. In the solid-phase assays, the overall order of sensitivity to wastes was Microtox > *D. magna* > *F. candida* reproduction > *F. candida* survival (Figure 1). In aqueous eluate assays, sensitivity was rated as *F. candida* reproduction > Microtox > *F. candida* survival. In methanol eluate assays, bioassay sensitivity was rated as Microtox > *F. candida* reproduction > *F. candida* survival, with a close range from both the *F. candida* endpoints. In the dichloromethane eluate assays, the same ranking was observed, but with more definite differences between *F. candida* reproduction and survival.

3.3. Solid-phase and eluate toxicity comparison

3.3.1. F. candida *reproduction assay*

No significant reduction in survival was detected with the addition of solvent in eluate assay controls compared to the controls of solid-phase assays (data not reported). A significant effect was only found for reproduction in the dichloromethane assays. However, this might be mainly due to intrinsic variability in reproduction in this species (Crouau and Cazes 2003) rather than a toxic effect of the solvent.

Survival and reproduction were sensitive to solid-phase wastes and eluates, reproduction being affected at lower concentrations than survival (Table 4). Concerning toxicity differences between wastes, the only general trend was the lower toxicity of the sludge composts for both endpoints and for all pollutant fractions. It is noteworthy that in composts survival was not inhibited, or inhibition was below 50% in all the eluate assays. Furthermore, the aerobic thermally dried sludge did not inhibit neither survival nor reproduction when applied as a dichloromethane eluate, despite its high toxicity in the solid-phase assays.

Survival in the solid-phase was positively correlated with that in the aqueous eluate tests ($r = 0.927$, $p=0.023$), and also with reproduction in the aqueous and methanol eluate tests ($r =0.831$ $p = 0.041$, and $r = 0.770$ $p = 0.043$, respectively). On the contrary, there was no correlation between effects on survival and reproduction in the solid-phase tests. Effects on reproduction in the solid-phase and in the eluate tests were not correlated, except for aqueous and methanol eluates, for whom a strong positive correlation ($p=0.006$) was found.

3.3.2. Microtox acute toxicity assay

V. fischeri was sensitive to solid-phase and eluates of the different wastes, as it provided meaningful toxicity values for each waste (Table 4). However, the relative toxicities of wastes were not coherent among the different pollutant fractions. Sludge compost toxicity was markedly lower than the remaining wastes in solid-phase eluates and methanol eluates, although this trend disappeared in the other eluates. On the contrary, in the aqueous eluates toxicity was similar for different wastes.

3.3.3. D. magna *acute toxicity assay*

Mobility inhibition in *D. magna* was also sensitive to the different wastes (Table 4), sludge composts being the least toxic. Anaerobic dewatered sludge exerted moderate toxicity and anaerobic thermally dried and dewatered sludge was the most toxic.

3.6. Pollutant concentrations and biological response

No correlation between toxicity and individual pollutant concentrations or pollutant groups could be detected, neither in solid-phase nor in aqueous and dichloromethane eluates. In contrast, some correlations appeared in the methanol eluate assays. More precisely, reproduction of *F. candida* was inversely correlated with copper levels ($r = -0.818$, $p = 0.024$), and the sum of heavy metals ($r = -0.788$, $p = 0.035$). Also luminescence in the Microtox assay was inversely correlated with the sum of organic pollutants ($r = -0.781$, $p = 0.038$), and the sum of non persistent organics ($r = -0.782$, $p = 0.038$).

3.7. Physicochemical properties of wastes and biological response

For *F. candida* assays, some physicochemical parameters of the original wastes were correlated with toxicity. In the solid-phase assays, survival was highly and positively correlated with the degree of waste stabilization ($r = 0.952$, $p = 0.001$), and negatively correlated with their total nitrogen ($r = -0.972$, $p<0.001$), hydrolysable nitrogen ($r = -0.97$, $p<0.001$), and ammonium content ($r = -0.786$, $p = 0.036$). This pattern was also observed for survival in the aqueous eluate assays, as there was a positive correlation between survival and degree of stabilization ($r = 0.906$, $p = 0.034$), as well as a negative relationship between survival and total nitrogen ($r = -0.941$, $p = 0.017$), and hydrolysable nitrogen ($r = -0.978$, $p = 0.004$). A negative correlation was also found

between survival in the methanol eluate assays and the total nitrogen ($r = -0.939$, $p = 0.002$).

No correlations were found between reproduction and waste physicochemical properties in the solid-phase assays. Nevertheless, negative correlations were observed between reproduction in both aqueous and methanol eluate assays and the ammonium concentration in the original wastes ($r = -0.904$ $p = 0.013$, and $r = -0.013$ $p = 0.002$ respectively), as well as with hydrolysable nitrogen in the aqueous eluate tests ($r = -0.835$, $p = 0.039$).

Similar correlations were also found in the *D. magna* assay, as mobility was positively correlated with waste stability ($r = 0.950$, $p = 0.004$), and negatively correlated with the total nitrogen ($r = -0.944$, $p = 0.005$) and hydrolysable nitrogen ($r = -0.927$, $p = 0.008$). On the contrary, no significant correlations were found between the Microtox assays and the physicochemical properties of the wastes.

4. DISCUSSION

4.1. Comparison of waste bioassays

Developing bioassays to assess the safety of polluted organic wastes application to soil is a priority in Europe, given the increased production of these materials and concern about this practice. Selection of such bioassays should primarily be based on their ecological relevance, but also on low experimental and economic costs.

The interest in bioassays has arisen due to the complex pollutant burden of wastes, but also because of the limitations of chemical methods to estimate the risk for ecosystems (Crouau et al. 2002). First, chemical methods require a previous knowledge of the substance groups to be analyzed and, therefore, not all potentially noxious chemicals are

394 monitored. Second, screening the most potentially noxious chemicals is too expensive,
395 given that thousands of them can be present in a polluted substrate. Third, chemical
396 methods give information about the total pollutant burden but not about its
397 bioavailability or release of end products. Finally, chemical methods do not detect
398 synergisms and antagonisms between chemicals. Bioassays overcome these limitations,
399 providing more realistic information on the potential effects of pollutants on living
400 organisms, however they do not provide information on the identity of chemicals
401 causing the effects measured (Brack 2003).
402 The growing concern for the prediction of the environmental hazard of wastes has led to
403 the development of standardized protocols which measure the harmful properties of
404 wastes from their leachates by using a combination of chemical analyses and aquatic
405 ecotoxicology assays. Nevertheless, most of this work has been limited to characterizing
406 inorganic wastes (Vaajasaari 2005). An example is the European standard EN 14735
407 (CEN 2005) for preparation of waste samples for ecotoxicity tests, applicable both to
408 inorganic and organic wastes. In this protocol, steps from sampling waste to the
409 performance of the biological tests, either using its solid-phase or its water eluates are
410 described. Furthermore, a list of potential terrestrial and aquatic organisms to be used is
411 proposed. Results of the present study demonstrate the sensitivity to wastes of some of
412 these bioassays, and despite the generalized lack of coherence among them, their
413 differential sensitivity is shown. Microtox toxicity was not correlated with any of the
414 remaining bioassays and neither was collembolan reproduction. However, mortality of
415 *F. candida* in solid-phase was highly correlated with inhibition in the mobility of *D.*
416 *magna.*
417

418 **4.2. Solid-phase versus eluate assays in waste testing**

Direct measure of waste toxicity for terrestrial soils and sediment organisms (solid-phase tests) should be the most relevant way to estimate its ecotoxicological potential, as it is close to real situations. However, only some works have taken this approach for wastes (Crouau et al 2002, Domene et al. 2007, Krogh and Pedersen 1997, Krogh et al. 1997, Pandard et al. 2006, Robidoux et al. 1998, Renoux et al. 2001), and most of the studies have been performed with aqueous eluates or leachates using as test organisms: microorganisms (Fuentes et al. 2006, Mantis et al. 2005, Park et al. 2005, Robidoux et al. 1998), plants (Fuentes et al. 2006, Garcia et al. 1991, Renoux et al. 2001, Robidoux et al. 1998, Tiquia and Tam 1998) or daphnids (Fjällborg and Dave 2003, Fjällborg et al. 2005, Molina-Barahona et al. 2005).

Some authors have criticized the use of eluates to extrapolate waste effects on soils because eluates only give information on the pollutants instantaneous bioavailability, not reflecting their longer-term bioavailability (McMillen et al. 2003). Furthermore, several confounding phenomena usually hinder finding correlations between pollutant concentrations in eluates and biological responses, making the interpretation of results difficult (Alexander et al. 2003, McMillen et al. 2003). More precisely, when eluates are used in aquatic tests or terrestrial soils after being mixed with a clean soil, synergic or antagonistic effects between pollutants or changes in their bioavailability may strongly modify the eluates' toxicity with respect to that of the original soils or wastes.

Solid-phase waste assays are, without question, more relevant than eluate assays mainly because they are the closest to real situations. However, solid-phase assays incur several problems in their practical application (Domene et al. 2007). First, organic matter represents a significant percentage of organic wastes, and can mask and underestimate their potential toxicity in short-term bioassays through pollutant sorption and nutritious effects on soil fauna. Second, wastes' physicochemical properties and water holding

444 retention capacity usually are very different to those in the test substrate, a fact that may
445 generate wide variations in these parameters depending on test concentrations. Indeed
446 test concentrations may influence the chemicals' bioavailability or the response of
447 organisms to their toxic effects. Third, solid-phase bioassays do not allow the
448 identification of the pollutant fraction mainly explaining the observed toxic effects,
449 while a combined testing of different eluates (aqueous and organic solvents) may give
450 indications on the pollutant fraction mainly contributing to the harmful effects exerted
451 by organic wastes. Finally, eluate assays may reduce the experimental effort needed for
452 the bioassays, since some steps of sample preparation for solid-phase testing could be
453 omitted (namely drying and grounding, but especially all work associated with the
454 monitoring of wastes' physicochemical properties and their water holding capacities).
455 Therefore, the choice of method is not an easy one, since both solid-phase and eluate
456 approaches imply significant limitations. Furthermore, the number of works comparing
457 toxicities obtained through solid-phase assays with terrestrial organisms and eluate
458 assays with aquatic organisms are still very scarce, although they concord in not finding
459 significant correlations (Loureiro et al. 2005, Sheehan et al. 2003).
460 Results from this study indicated aqueous eluates were representative of the solid-phase
461 acute toxicity to *F. candida*, but also to that of *D. magna*. Despite this, the lack of
462 correlation between *F. candida* reproduction in the solid-phase and in the aqueous
463 eluate tests suggest that even aqueous eluates are not suitable for estimating chronic
464 effects. This is consistent with several authors who claim that toxicity results obtained
465 from aquatic tests using soil eluates can only be extrapolated to soils for short-term
466 assays focused on lethal endpoints (McMillen et al. 2005), and that a proper assessment
467 of the long-term ecological risk should be based on chronic assays using soil organisms
468 and sublethal endpoints (van Gestel et al. 2001). Incoherencies between the chronic

toxicity results obtained for *F. candida* and those from the Microtox assay, stress the lack of representativeness of the aquatic tests with waste eluates to predict effects on soils. This finding agrees with the work of Sheehan et al. (2003), who failed to find any correlation between toxicity of several polluted soils, their leachates and their corresponding groundwaters, using terrestrial tests (*Eisenia fetida* survival) and aquatic tests (Microtox test, and daphnids immobilization test). Loureiro et al. (2005), comparing results of different bioassays with two polluted soils and a control soil, also did not find any correlation of results between the *F. candida* reproduction test and aquatic test results (Microtox luminescence test, and *Daphnia magna* immobilization and reproduction test). On the contrary, Microtox results have been correlated with fish lethality in assays with wastes from a petroleum refinery (Park et al. 2005).

4.3. Main contributors to organic waste toxicity

Both in the solid-phase and eluate assays, composted sewage sludges presented lower toxicity than the other wastes (with the exception of aqueous and dichloromethane eluates in Microtox). However, the battery of tests did not determine the most toxic wastes, as it depended both on test species and pollutant fraction. These results demonstrate the influence of the species' intrinsic sensitivity to a given chemical burden on the observed toxicities, and also the compulsory requirement of test batteries including several organisms for a proper ecotoxicological risk assessment of wastes.

Results from this study did not show correlation of toxicities with pollutant burden neither in the solid-phase, aqueous or dichloromethane eluate assays, in agreement with reports by other authors regarding polluted soils and their corresponding eluates (Sheehan et al. 2003). However, some correlations were ostensible in methanol eluate assays, since the sum of heavy metals explained the reproduction inhibition in *F.*

494 *candida*, while the sum of all the organic pollutants and the sum of non persistent
495 organics explained the inhibition of luminescence in the Microtox assay. The
496 relationship found for heavy metals in *F. candida* is not surprising, as most of the heavy
497 metal burden in sludge is not water soluble but is adsorbed to the organic matrix
498 (Alonso et al. 2006), which can be partly solubilized by methanol.
499 On the contrary, several physicochemical parameters of the original wastes related to
500 organic matter stability were highly explanatory for the acute toxicity observed in *F.*
501 *candida* survival and *D. magna* mobility in the solid-phase and aqueous eluate assays.
502 Furthermore, non-hydrolysable nitrogen and ammonium concentration in the original
503 wastes also accounted for inhibition in reproduction of *F. candida* in water eluates,
504 while ammonium was the inhibiting factor in methanol eluate assays. Non stabilized
505 wastes are easily decomposed, as an important percentage of their organic matter is
506 labile, mainly polysaccharides and proteins (Rovira and Vallejo 2002). Along the
507 decomposition process, the percentage of stable organic matter increases, while there is
508 a loss of total nitrogen by depletion of its more hydrolysable (labile) fraction, mainly as
509 ammonia releases (Grube et al. 2006, Martins and Dewes 1992, Witter and Lopez-Real
510 1988). In addition to ammonium, other noxious secondary metabolites of decomposition
511 are released, like phenols and organic acids (Déportes et al. 1995, Fang et al. 1999,
512 Garcia et al. 1991, Huang et al. 2004, Mathur et al. 1993). Hence, the more stabilized an
513 organic waste, the higher is the percentage of stable organic matter, and the lower the
514 levels of total nitrogen, hydrolysable nitrogen, and ammonium. Furthermore, with
515 decomposition, waste toxicity may decrease because non-persistent organic pollutants
516 can be degraded by microorganisms (Abad et al. 2005, Bagó et al. 2005, Déportes et al.
517 1995, Marttinen et al. 2004, Sanz et al. 2006), and because pollutant bioavailability is
518 the lowest in the most stabilized wastes (Fuentes et al. 2006). This relationship between

519 waste stability and toxicity has already been reported for plants (Pascual et al. 1997,
520 Zmora-Nahum et al. 2005), but is scarcely documented for soil fauna (Neher 1999).
521 Garcia et al. (1991) already stressed the relevance of organic wastes' aqueous eluates to
522 indicate the wastes' maturity and phytotoxicity (Fuentes et al. 2006). Other authors have
523 also found this association using the Microtox assay (Fuentes et al. 2006, Walter et al.
524 2006). Particularly, Tiquia and Tam (1998) showed that increasing the stability of pig
525 manure composts decreases ammonium and metal concentrations in eluates, and
526 reduces phytotoxicity.
527
528
529 **CONCLUSIONS**
530
531 The general lack of coherence of results between bioassays, shows the varied sensitivity
532 of different test organisms, and the need to use test batteries for a proper
533 ecotoxicological risk assessment.
534 Waste eluates were not representative of the chronic toxicity exerted by solid-phase
535 waste. However, aqueous eluates exerted equivalent acute toxicity to that of solid-phase
536 waste in collembolans and daphnids. Therefore, extrapolation to soils of waste toxicity
537 results obtained from aquatic tests using aqueous soil eluates should be only acceptable
538 for short-term assays focused on lethal endpoints.
539 Chemical methods are not suitable for a proper risk assessment of wastes, as no
540 correlation of toxicities with pollutant burden were found neither in the solid-phase, nor
541 in aqueous or dichloromethane eluate assays. The only direct relationships were found
542 in methanol eluate assays between total heavy metal burden and collembolan chronic

toxicity, and between the total organic and non-persistent organic burden with the Microtox assay.

Physicochemical parameters related to organic matter stability were highly explanatory of the acute toxicity observed in collembolans and daphnids in the solid-phase and aqueous eluate assays, but also of the chronic toxicity for collembolans in aqueous and methanol eluates. Ammonium and other water-soluble decomposition end products might be the main explanation for this association.

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FIGURE CAPTIONS

Figure 1. Bioassays comparison of inhibition values in solid-phase assays of the different wastes. Values were log-transformed and expressed in g Kg^{-1} in *F. candida* assay, and g L^{-1} in *D. magna* and Microtox assays.

Table 1. Origin, treatments and post-treatments of the organic wastes (AE = aerobically digested sewage, AN = anaerobically digested sewage, SL = pig slurry, D = dewatered, C = composted, T = thermally dried).

Waste	Origin	Treatment	Post-treatment
AED	Banyoles WWTP	Aerobic digestion, dewatering	None
AEC	Banyoles WWTP	Aerobic digestion, dewatering	Composting in vessel
AET	Banyoles WWTP	Aerobic digestion, dewatering	Thermal drying
AND	Blanes WWTP	Anaerobic digestion, dewatering	None
ANC	Blanes WWTP	Anaerobic digestion, dewatering	Composting in heap
ANT	Blanes WWTP	Anaerobic digestion, dewatering	Thermal drying
SLT	Juneda WTP	Anaerobic digestion, dewatering	Thermal drying

Table 2. Physicochemical properties of the wastes studied (from Domene et al. 2007).

Parameter	Units	AEC	AED	AET	ANC	AND	ANT	SLT
Dry matter	g Kg^{-1} (w/*w.w.*)	449	150	945	470	199	844	865
WHC	% (*w/w.w.*)	74.4	63.9	74.7	64.9	64.8	67.9	55.9
pH	water, 1:5 (v/v)	7.8	8.1	6.9	7.2	8.4	7.2	6.4
Electrical conductivity	dS/m, 25ºC	1.2	1.5	3.57	4.2	2.25	6.22	64.65
Organic matter	g Kg^{-1} (*d.w.*)	622	684	687	551	566	668	612
Stable organic matter	%	50.1	37.8	40.4	54.2	47.7	46.7	36.6
N	g Kg^{-1} (*d.w.*)	39.5	62.4	60.6	23.7	38.8	53.3	62.5
Non-hydrolysable N	g Kg^{-1} (*d.w.*)	17.0	16.4	19.1	16.1	12.4	18.4	10.9
Hydrolysable N	g Kg^{-1} (*d.w.*)	22.5	46.0	41.5	7.6	26.4	34.9	51.6
NH_4-N	g Kg^{-1} (d.*w.*)	2.7	14.0	8.0	3.4	15.1	11.6	52.9
P	g Kg^{-1} (*d.w.*)	22.0	20.4	20.5	28.6	33.6	29.2	20.4
K	g Kg^{-1} (*d.w.*)	3.6	1.9	2.2	4.4	2.3	2.5	55.1

Table 3. Solid-phase and eluates pollutant burden. Values for the solid-phase are expressed in mg Kg^{-1} except for of PCB (in ng g^{-1}), and for PCDD/F (in ng TE Kg^{-1}). For eluates, concentrations are expressed in the same units, but are in reference to the total mass of dry sludge used for the extraction. *nd* = non-detectable levels.

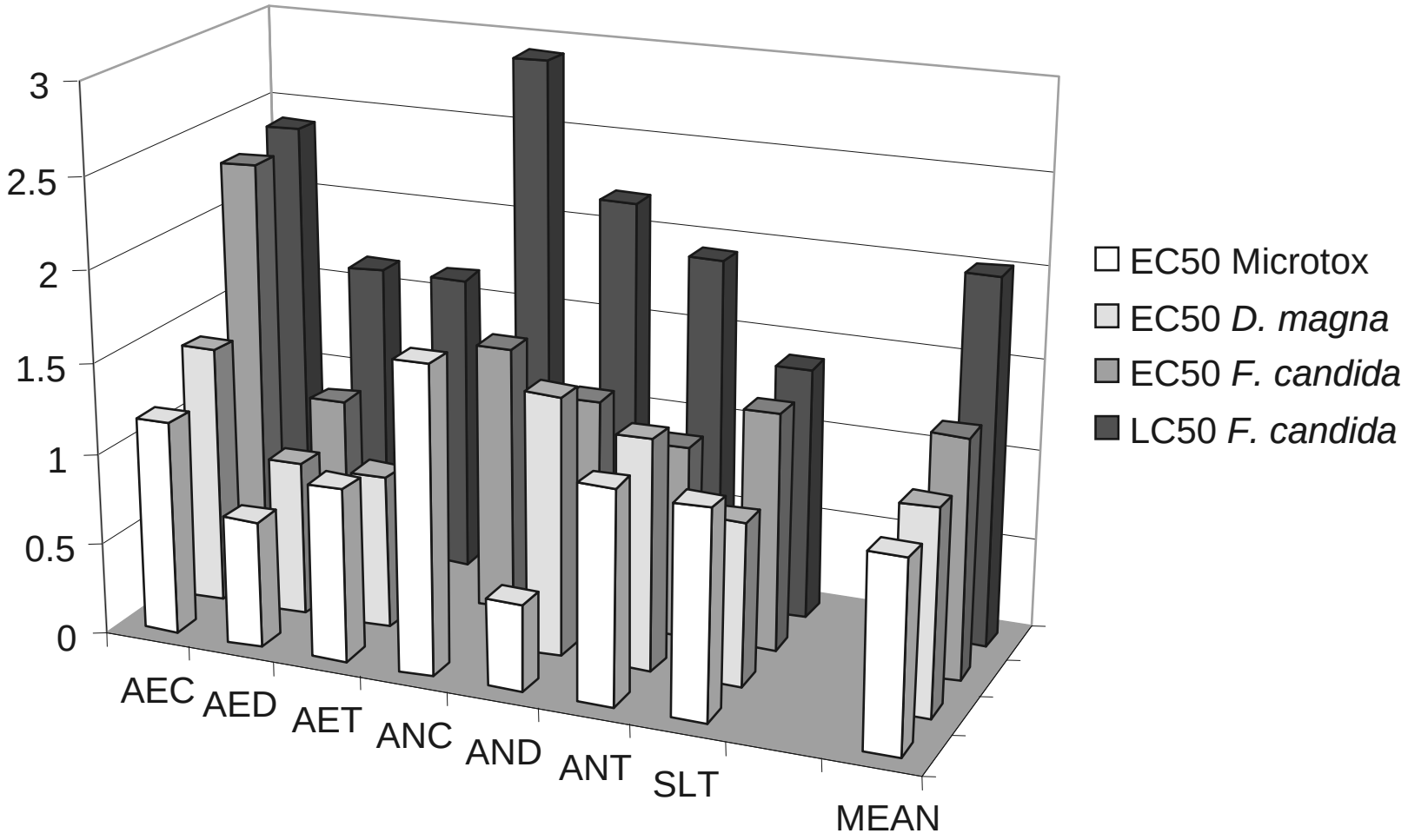
Assay	Pollutant	AEC	AED	AET	ANC	AND	ANT	SLT
Solid-phase	Cd	1	1.3	1.3	3.5	3.2	3.1	<0.7
	Cr	345	55	30	53	54	127	15
	Cu	294	624	645	798	933	833	780
	Hg	0.67	1.33	0.95	2.13	2.51	2.25	0.12
	Ni	59	80	53	76	64	45	29
	Pb	1196	3940	3747	92	78	85	<20
	Zn	843	956	952	1028	988	890	2060
	DEHP	10	61	27	22	143	71	1
	LAS	298	816	331	214	3240	5572	60
	NPE	86	153	76	158	513	573	54
	PAH	0.1	0.4	0.3	1.6	1.1	1.4	0.05
	PCB	15	34	29	41	23	29	<7
	PCDD/F	16	15.6	13.7	12.4	7.7	13.2	0.3
Water eluate	Cd	0.33	1.17	1.36	2.13	nd	0.72	nd
	Cr	0.63	10.07	0.78	3.96	nd	1.75	nd
	Cu	0.13	3.46	0.63	1.49	nd	1.56	nd
	Hg	0.01	0.18	0.77	0.47	nd	0.01	nd
	Ni	0.05	0.73	10.33	2.57	nd	0.1	nd
	Pb	0.24	2.57	24.3	3.46	0.02	0.04	nd
	Zn	0.11	1.43	28.7	22.6	0.02	0.2	nd
	DEHP	nd	nd	nd	nd	nd	nd	nd
	LAS	nd	nd	nd	nd	nd	nd	nd
	NPE	nd	nd	nd	nd	nd	nd	nd
	PAH	nd	nd	nd	nd	nd	nd	nd
	PCB	nd	nd	nd	nd	nd	nd	nd
	PCDD/F	nd	nd	nd	nd	nd	nd	nd
Methanol eluate	Cd	nd	nd	nd	nd	nd	nd	nd
	Cr	0.07	1.41	0.09	0.09	0.09	0.19	0.06
	Cu	0.23	3.27	1.64	0.25	0.17	2.4	16.6

	Hg	nd	nd	nd	nd	nd	nd	nd
	Ni	0.13	4.5	0.55	nd	nd	0.08	0.65
	Pb	nd	2.4	0.23	nd	nd	nd	nd
	Zn	0.24	8.55	0.33	0.38	0.06	0.38	16.28
	DEHP	8.76	27.2	39.6	24.1	115.9	54.2	0.49
	LAS	0.19	0.15	0.03	0.09	4.25	4.66	0
	NPE	36	62	30	53	237	296	77
	PAH	0.05	0.09	0.12	0.86	0.27	0.43	0.01
	PCB	nd	nd	nd	nd	nd	nd	nd
	PCDD/F	nd	nd	nd	nd	nd	nd	nd
Dichloromethane eluate	Cd	nd	nd	nd	nd	nd	nd	nd
	Cr	0.54	1.36	0.1	0.05	0.65	0.23	nd
	Cu	0.25	0.99	0.29	0.26	1.2	0.91	0.81
	Hg	nd	nd	nd	nd	nd	nd	nd
	Ni	0.25	0.24	0.05	nd	0.06	0.04	nd
	Pb	0.38	9.39	1.13	nd	0.18	0.1	nd
	Zn	0.55	1.58	0.27	0.05	1.17	0.73	0.26
	DEHP	10.4	55.0	27.8	175.3	183.5	368.8	6.13
	LAS	nd	0.03	nd	nd	0.09	0.06	nd
	NPE	36	62	32	59	154	215	25
	PAH	0.09	0.04	0.09	0.44	0.25	0.32	0.01
	PCB	nd	nd	nd	nd	nd	nd	nd
	PCDD/F	nd	nd	nd	nd	nd	nd	nd

Table 4. Toxicity values for each waste in the different bioassays. Values of solid-phase assays are expressed as g Kg^{-1} for *F. candida* assay, as $g \cdot L^{-1}$ for *D. magna* assay, and as $mg \cdot L^{-1}$ for Microtox. In the eluate assays, concentration values are expressed based on the mass of waste initially extracted.

Assay	Waste	*F. candida* LC50	*F. candida* EC50	*D. magna* EC50	Microtox EC50
Solid-phase	AEC	252.3 (221.7, 287.2)	207 (36.8, 1142)	25.4 (21.4, 30.6)	13.9 (9.2, 21.7)
	AED	43.9 (34.1, 56.6)	10.0 (3.8, 23.8)	6.1 (4.8, 7.7)	4.4 (2.46, 7.91)
	AET	44.0 (37.4, 51.7)	5.3 (2.8, 9.4)	6.1 (4.9, 7.5)	7.9 (6.5, 9.5)
	ANC	834 (626, 1110)	28.7 (17.7, 46.0)	-	47.9 (29.8, 88.9)
	AND	154 (134, 178)	16.4 (14.7, 18.2)	25.8 (21.7, 31.1)	1.61 (1.09, 2.4)
	ANT	85.6 (72.3, 101)	10.4 (7.5, 14.2)	17.6 (14.2, 21.7)	14.2 (12.3, 16.5)
	SLT	23.7 (20.2, 27.8)	19.4 (3.8, 86.4)	6.8 (5.9, 8.6)	12.5 (9.5, 16.6)
Water eluate	AEC	-	28.6 (11.8, 67.5)		75.4 (56.4, 100.8)
	AED	138 (111, 172)	4.0 (2.9, 5.6)		21.7 (16.4, 28.8)
	AET	245 (207, 290)	7.4 (3.6, 14.1)		65.0 (29.2, 281.5)
	ANC	-	-		-
	AND	628 (527, 750)	8.5 (3.5, 19.1)		60.3 (7.7, 486.3)
	ANT	279 (233, 334)	2.5 (1.6, 3.8)		97.3 (47.9, 204.7)
	SLT	129 (106, 129)	0.7 (0.3, 1.2)		54.6 (32.4,97.6)
Methanol eluate	AEC	-	656 (390, 1105)		23.7 (21.2, 26.4)
	AED	80.7 (68.5, 95.1)	24.6 (19.8, 30.4)		1.9 (0.9, 3.7)
	AET	254(312, 206)	230 (184, 288)		3.6 (3.1, 4.2)
	ANC	-	548 (283, 1061)		16.2 (13.7, 19.1)
	AND	100 (85.1, 118)	65.5 (56.0, 76.6)		1.0 (0.2, 4.7)
	ANT	87.9 (70.8, 109)	31.3 (20.4, 47.6)		1.57 (1.0, 2.5)
	SLT	311 (234, 413)	8.2 (5.4, 12.0)		10.8 (5.8, 16.2)
Dichloromethane eluate	AEC	-	-		4.3 (3.6, 5.3)
	AED	257 (225, 292)	72.0 (24.0, 212.7)		7.9 (3.0, 20.5)
	AET	-	-		2.1 (1.3, 3,4)
	ANC	-	93.5 (44.6, 194.8)		14.4 (11.3, 18.5)
	AND	133 (110, 161)	85.4 (31.0, 232.0)		0.8 (0.6, 1.5)
	ANT	248 (210, 291)	58.9 (39.1, 88.3)		2.4 (1.8, 3.3)
	SLT	254 (204, 316)	166 (108, 256)		5.4 (3.8, 7.7)

Figure 1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EC50 F. candida
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