Influence of Soil Properties on the Performance of *Folsomia candida*: Implications for Its Use in Soil Ecotoxicology Testing

Xavier Domene*†, Sónia Celinho‡, Paolo Campana§, Tiago Natal-da-Luz‡, Josep M. Alcañiz†, Pilar Andrés†, Jörg Römbke||, Paulo Sousa‡

† CREAF, Department of Animal and Plant Biology and Ecology - Autonomous University of Barcelona, Cerdanyola del Vallès, Spain

§ IMAR- CMA - Marine and Environmental Research Center, Department of Life Sciences - University of Coimbra, Coimbra, Portugal

§ Facoltà di Agrária - Università degli Studi di Torino, Grugliasco, Turin, Italy

|| ECT Oekotoxikologie GmbH, Flörsheim, Germany

*To whom correspondence may be addressed (x.domene@creaf.uab.es).

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Abstract: Nineteen Mediterranean natural soils with a wide range of properties and the Organization for Economic Cooperation and Development (OECD) artificial soil were used to assess the influence of soil properties on the results of avoidance and reproduction tests carried out with the soil collembolan species *Folsomia candida*. Compared to natural soils, the OECD soil was mostly rejected by individuals when a natural soil was offered in avoidance tests, and the number of offspring produced was generally lower than the one obtained in natural soils. None of the soil properties assessed showed a significant influence on the avoidance behavior. More precisely, only soil moisture was included in the model explaining the avoidance response (avoidance increased with increasing differences in moisture), but its contribution was marginally not significant. The model derived explained only 16% of the variance in avoidance response. On the contrary, several soil properties influenced significantly reproduction (number of offspring increased with increasing moisture content, increasing coarse texture and decreasing nitrogen content). In this case, the model explained 45% of the variance in reproduction. These results, together with the fact that most of the selected soils fulfilled the validity criteria in both avoidance and reproduction tests, confirm literature experiences showing that this species is relatively insensitive to soil properties and hence highly suitable to be used in ecotoxicological tests with natural soils. In addition, our study highlights the need for accuracy in soil moisture adjustment in soil ecotoxicological tests with this species. Otherwise, results of both avoidance and reproduction tests might be biased.

Keywords–Behavioral toxicology, Ecotoxicology, Terrestrial invertebrate toxicology

INTRODUCTION

Most soil ecotoxicological tests are carried out using the artificial soil developed by the Organization for Economic Cooperation and Development (OECD) and the standard German soil LUFA 2.2 [1], which allow comparisons between laboratories as well as between different species [2]. However, the use of standardized soils does not represent realistic field conditions, since soil properties can have an important influence on the bioavailability and toxicity of soil pollutants. The use of natural soils in the ecotoxicological risk assessment of pollutants, both in prospective and retrospective risk assessments, is always advisable if the aim is to generate representative data for real situations [3]. However, soil properties do influence the toxicity values observed and may lead to biased conclusions depending on the soil used. Equally important, soil properties itself influence the behavior and performance of the test species [3,4].

Reproduction is the most commonly used chronic endpoint in invertebrate ecotoxicology. This situation is caused by the fact that it is more sensitive than mortality, since even slight impacts caused by the test chemical can disturb biochemical or physiological processes which are able to be transferred through a cascade of events to effects on the reproductive outcome [5]. However, the use of reproduction as endpoint requires considerable effort in terms of time and handling, and this is why, in recent years, alternative endpoints which provide similar information with less experimental effort have been proposed. Especially in the last decade, avoidance tests have increasingly been used in soil ecotoxicity studies, most of the attempts being carried out with earthworms [6-10]. This led to the development of an earthworm test guideline for earthworms [11]. In 2010, the final draft of a test guideline with collembolans was developed [12]. Avoidance tests have been only scarcely used with soil organisms other than earthworms, e.g., enchytraeids [13-17], collembolans [8, 10, 18, 19], or isopods [20]. Avoidance tests are usually as sensitive as reproduction tests, but have a shorter duration, and are easier to perform than the existing acute or reproduction tests [6, 8, 9, 15, 21]. However, there are exceptions, e.g., sometimes avoidance can be less sensitive than reproduction [22].

Besides the widely known influence of soil properties on the toxic effect of chemicals through their influence on the chemical's bioavailability, some studies have also reported the influence of soil properties on the reproduction of collembolans [3, 13, 14, 16, 23-29]. However, less is known about their effects in avoidance tests [4].

The influence of soil properties itself on the results of ecotoxicological tests is removed by the expression of these results as percent of the response in controls (i.e., reproduction inhibition is expressed as % of that in the controls). However, in certain circumstances, when soil properties are far from the ecological requirements of the test species, the unsuitability of soil adds to toxic stress, magnifying the inhibition and overestimating the toxicity [29]. Also, when assessing the risk of pollutants to soil organisms, the properties of the selected reference soil (or different soils if a multi-reference approach is adopted), might cause biased conclusions, since it is usually a soil found nearby or a standard soil. In particular this happens if the properties of the reference soil differ clearly from the test soil [30, 31]. In theory, such a case should not happen – but it might be inevitable when no uncontaminated regional reference soil is available.

Hence, the accurate choice and preparation of the reference soil is of utmost importance. Preferably it should be the same (or at least similar) soil as tested but without contamination.

Equally important, the reference soil should satisfy the biological requirements of the test species. However, studies focusing on the influence of soil properties on the performance of test species in soil ecotoxicology are still scarce. This study aims to contribute to fill this gap by assessing the influence of soil properties on the avoidance behavior and the reproduction of the soil collembolan *Folsomia candida*. This species is one of the most commonly used test organisms in soil ecotoxicological studies; partly because standard guidelines are available [32,33]. Using a wide set of natural soils, a predictive model as tool for the evaluation of the influence of soil properties on the performance of this species was developed. The model development was a generalized multiple regression analysis of the multivariate soil properties removing insignificant parameters and ending up with one factor for the avoidance tests and six factors for the reproduction tests. Moreover, the results from our study might also be useful for the selection of new reference soils for ecotoxicological testing in Europe, since no reference soils were selected so far from the Iberian Peninsula [34, 35].

METHODS

Soils selected and test species

All test soils came from three European Mediterranean regions: Alentejo (Portugal), Catalonia (Spain), and Liguria (Italy). Sampling was mainly performed at agricultural sites, but in a few cases samples were taken at grassland, shrubland or forest sites (**Table 1**). At the agricultural sites, no or only low agrochemical impact did happen. The available pedological information in each region was used to select the soils [36-39]. The main criterion was to cover a broad range of soil properties. In addition, OECD artificial soil was prepared according to the OECD Guideline 207 [40]. It was used as an additional, external control, performed to assure a lasting and similar reaction of the test organisms.

Topsoil samples (0-20 cm depth) were collected, 5-mm sieved, and air-dried. Then soils were defaunated using two alternating freezing-thawing cycles, each consisting on placing soils at -20ºC for 4 d followed by 4 d at 20ºC. Several soil properties were analysed: pH and water holding capacity (WHC) [41], texture [42], organic carbon [43], total nitrogen [44], cation exchange capacity (CEC) define all acronyms at first meniton [45], moisture (expressed as weight loss per soil dry wt after drying at 105ºC for 12 h). In addition, heavy metals were measured according to [46] in order to discard pollution that might affect the outcomes of the bioassays regarding data reported from [47]. In It2 and UAB can the name of the soil be spelled out? soils, copper was present at relatively higher concentrations than the usual background levels. This fact, mostly explained the use of copper sulfate in traditional vineyard cultures, are not expected to affect the results of the tests since the bioavailability of copper was probably low because of aging processes [48], and because the performance of collembolans in these soils was acceptable according to ISO 11267 [32]. The 6.7% organic matter content in the OECD soil is slightly below to the expected 10% content, something that can only be attributed to the subsample taken for the analysis or to peat recalcitrance to the oxidation method used.

The collembolan *Folsomia candida* (Isotomidae: Collembola) was used as test species, obtained from cultures of the Laboratory of Soil Ecology and Ecotoxicology of the University of Coimbra (Portugal). The animals were kept in vessels filled with a wet mixture of plaster of Paris and charcoal (9:1, w/w), in a climatic chamber at constant temperature of 20±2ºC and with a 16:8 h (light:dark) photoperiod. Individuals were fed weekly with granulated dry yeast.

Soil preparation

The water content of the test soils was adjusted in a way that each soil stayed moist and crumbly. This suitable moisture content was generally around 40 to 60% of the maximum water holding capacity (WHCmax) (Table 1). However, this range was slightly exceeded in a very sandy soil (Pz, 63%), and was sometimes not achieved in the more clayey and silty soils (Br, Cam, Gan, Gra, Pra, Riu, Pz, and OECD), for which the water content was adjusted to approximately 30 to 40% of the WHC, since higher water contents originated a doughy soil structure.

Avoidance tests

Avoidance tests were performed according to the standardized draft guideline for collembolans (ISO 17512-2 draft, [12]). Avoidance tests were carried out using some of the soils (Br, Gan, Gra, It2, It3, It4, Lit, Luv, OECD, Por, Pra, Pz, and Riu) comparing pairs of soils within the same region. Each replicate consisted of a polyethylene cylindrical container (7 cm diameter x 6 cm height) horizontally divided in two equal sections with a removable plastic sheet. Each section was filled with 30 g wet soil: one section was filled with a control soil and the other with test soil. Then, 20 individuals (10 – 12-d old) were transferred to the center of the container, and left under controlled climatic conditions for 48 h (20±2ºC and 16:8 h light:dark photoperiod). At the end of this period, the soil from each section was taken separately, poured into a 200-ml Erlenmeyer flask and flooded with water. Soil was gently stirred in order to force the individuals to float on the water surface and enable counting.

Two different avoidance assays were carried out: ~~a)~~ dual-control tests, where both sections of the test vessels were filled with the same soil, with the aim of determining if the individuals' distribution was affected by factors other than soil properties [6, 30]; b) avoidance tests, where all the possible combinations of different soils within each region were compared (control soil in the left section, test soil in the right section). (delete numbered or lettered lists, it is not journal style) The aim in these assays was to determine the influence of soil properties on the avoidance behavior in this species. Both for the control-dual tests and the avoidance tests, 10 replicates per each comparison were prepared. Due to sample limitations, but in accordance to the protocol ISO [12], only 5 replicates were used for combinations with the soils Gan, Gra, Por, Pra, and Riu. This corresponded in total to 105 dual-control test replicates and 240 test avoidance test replicates.

Reproduction tests

Reproduction was determined in the nineteen natural soils and in the OECD soil (Table 1) according to ISO 11267 [32]. Ten replicates were prepared for each soil, only five replicates in the soils Cam, Coll, and Vil, due to sample limitation. Each replicate consisted of a wet soil (corresponding to 30 g dry wt) in a sealed 150 ml glass flask. In total, 174 test vessels were prepared. The test was run for 28 d under constant climatic conditions (21±1ºC and 16:8 h light:dark photoperiod). At the start of the test and after two weeks, 3 mg of granulated yeast were added to each replicate. At the end of the test period, soil was poured into a 200 ml Erlenmeyer flask and flooded with water, followed by the addition of a dark dye. Afterwards, a picture of the surface was made, showing the individuals floating on the water, and the number of juveniles was counted by means of the image treatment software ImageTool 3.0 (University of Texas, Health Science Center, San Antonio, USA).

Data treatment

Soils comparison Is this a second or third level heading? If it is third, it should begin the sentence below with a period after comparison.

The soils were compared by means of a principal components analysis (PCA) using the software package SPSS 15.0 (IL, USA). The analysis included variables showing low levels of correlation (with Pearson's correlation coefficient <0.8), namely the maximum water holding capacity, pH (H_2O), coarse and fine sand, silt and clay contents, organic carbon, total nitrogen, and cation exchange capacity.

Avoidance tests

Significant differences in the distribution of individuals between both sides of the containers were determined by means of Fisher's exact test [49]. This procedure allows comparing the observed distribution of individuals with an expected distribution assuming no avoidance as null hypothesis, as described in Natal-da-Luz et al. [8]. For the dual-control tests, a two-tailed test was used, which assumes the null hypothesis of an equal distribution of individuals at both sides. For the avoidance tests with pairs of different soils, a one-tailed test was used, assuming as null hypothesis the lack of avoidance, i.e., that half of the total individuals tested remain in the soil being assessed (test soil). The null hypothesis was rejected for a probability equal or lower than 0.05. Statistical assessment was carried out taking only surviving individuals into account, without any correction of the mortality observed in control-dual tests, which was low in general (i.e., below 20%).

In order to evaluate which soil properties were mainly responsible for the avoidance patterns, the outcomes of avoidance tests were used. Namely, the rate of individuals in the test soil for each pair of soil combinations (*N*) does the N represent number? If so it should be formatted lowercase italic was calculated by the equation $N = T/(C+T)$, where T = number of individuals in the test soil, and C = number of individuals in the control soil. The *N* ranged from 0 (no individuals present) to 1 (all the individuals in the test soil). Data from combinations with OECD artificial soil was not used in this case, given their clearly different properties compared to natural soils (**Fig. 1**) and due to the high proportion of combinations using this soil, which might strongly bias the results. This reduced the sample size from n = 240 to 155. Further, using data from the remaining soil combinations, we calculated for each soil property the quotient (Q) between the value in the test soil divided by the value in the control soil. A value of Q<1 indicated lower values in the test soil for the respective soil parameter, a Q=1 indicated no differences, while any Q>1 indicated higher values for the respective parameter in the test soil compared to the control soil.

To relate avoidance with soil parameters quotients, we constructed a regression model through generalized linear models (GLM) using Brodgar 2.5.2 (Highland Statistics, Newburgh, UK). Outliers in the response variable, within each combination of soils, were assessed through boxplot graphics and were removed from further analysis. This procedure reduced the sample size slightly from n = 155 to n = 132. The explanatory variables showing high correlation were also not used for the model construction (those showing correlation coefficient>0.8 or variance inflation factor (VIF) >10). The explanatory variables retained were used for model construction assuming a binomial distribution and using logarithm as link function. After different trials, the model containing the variables with the best adjustment to our data was obtained, selected with an automatic backward selection procedure. Among the models considered, that with the lowest Akaike Information Criteria (AIC) value was accepted as the best model. Please check this sentence. The suitability of the model was evaluated by the assessment of the homogeneity of the residuals (looking to the residuals versus fitted values plot), and their normality (by means of a normal Q-Q-plot).

The avoidance tests were performed on separate dates. However, we assumed seasonal response variation in the test species due its parthenogenetic nature and the constant environmental conditions used both in cultures and tests.

Reproduction tests

Given the high number of comparisons that could be carried out and given the main interest of this study, we only compared reproduction in natural soils versus that in OECD artificial soil. Significant differences were verified by means of the Fisher's least significant difference (LSD) test using SPSS 15.0. In order to relate the soil properties to the reproduction outcome, we also constructed a multiple regression model by means of GLM. Data from tests with OECD soil were also not used in this analysis, given their clearly different properties compared to the natural soils (Fig. 1), which could strongly bias the results. This reduced the sample size from n=194 to 164. With data from the natural soils, we constructed a matrix containing each replicate as row, and the response variable (number of juveniles) and the different explanatory variables (soil parameter values) as columns. The outliers in the response variable within each soil type were also detected through boxplot graphics and removed, something that reduced the sample size to n=146. Again, the explanatory variables showing high correlation were not used for the model construction. For the model construction using GLM, and given the high overdispersion of the model when a Poisson distribution was assumed, we finally assumed a quasipoisson distribution, using logarithm as link function. The model with best adjustment to our data was obtained, assuming that the best fitting model had the lowest AIC value, which was achieved with an automatic backward selection procedure. The suitability of the model was also evaluated by the assessment of the homogeneity and normality of the residuals.

Despite of the fact that the tests were performed on separate dates, we assumed no seasonal response variation in the test species for the reasons already appointed.

RESULTS

Soils characterization

~~PCA~~ Principal component analysis (PCA) discriminated soils according to the three main axes that explained 38.3, 28.2, and 16.7% of the variance, respectively (Fig. 1). The first axis was positively associated with the pH (0.852) and clay content (0.809), and negatively associated with coarse sand (-0.815). The second axis mainly reflected the organic carbon (0.941) and total nitrogen contents (0.918). Finally, the third axis was mainly explained by the fine sand content (0.889). The soils located in the upper part of the cloud were generally fine textured soils with basic pH, and the soils located in the lower part being coarse sandy soils with more acidic pH. The peripheral position of the Sta soil is due to its higher organic carbon and total N content compared to the other soils, while the separated position of OECD is due to its elevated fine sand content.

Avoidance tests

Mortality in dual-control tests was below 20% (only in Pra/Pra it was slightly exceeded: 22%), and between 40 and 60% of the individuals were present in each section, fulfilling the validity criteria of the ISO 17512-2 [12]. Results from the dual-control tests indicated that the individuals were distributed at random among the two sections of the test containers according to the two-tailed Fischer's exact test) (**Fig. 2**). Hence, no other influence than soil properties appeared to explain any possible avoidance behavior.

In most of the avoidance tests using two different soils, the distribution of the individuals was not random, showing their sensitivity to any of the soil characteristics. More precisely, avoidance was observed in 74% of the combinations (**Fig. 3**). When compared with natural soils, the OECD artificial soil was avoided by collembolans in most of the combinations. However, OECD soil was preferred when compared with Pra soil, while an equal distribution on both sides was found when compared with Luv soil.

Most of the explanatory variables (soil quotients or Q) presented high correlation: Q_C with Q_N (0.98); Q_Moisture with Q_Clay, Q_CEC and Q_WHC_{max} (0.95, 0.94 and 0.86 respectively); Q_CEC with Q_Clay, Q_Silt and Q_ WHC_{max} (0.93, 0.84 and 0.81 respectively); Q_Coarsesand with Q_C (0.81); and finally Q_Clay with Q_ WHC_{max} (0.81). After the calculation of VIF values and the progressive removal of the variable with higher VIF value, a final set of eight variables was selected (all with VIF values below 10): Q_pH, Q_Finesand, Q_Silt, , Q_C, Q_moisture and Q_%WHC. The GLM of the rate of individuals in the test soil with respect to the soil properties quotients lead to an AIC=173.2 in the final model (compared to the AIC equal to 177.6 in the full model), which only contained Q_ WHC_{max}as significant variable (*p*=0.042) (**Table 2**). Hence, the final model was expressed as N=e^[(-0.8422)+(0.2287*Q_moisture))], where *N* lowercase italic? is the number of individuals in the test soil divided by the total number of individuals, and Q_moisture is the moisture of the test soil divided by that in the control soil.

The model derived provided a R^2 of 0.16 (where R^2=[(null deviance-residual deviance)/(null deviance)]), meaning that this model is able to explain only 16% of the variance of the avoidance response. In addition, the contribution of Q_moisture was marginally non-significant (*p*=0.07). All this indicate the low ability of the model to predict the avoidance response of this species.

Reproduction tests

In the reproduction tests, mortality was below 20% in almost all soils; only in Pra soil all individuals died. The reproduction of *F. candida* in natural soils generally differs significantly from that in OECD artificial soil in half of the cases. In OECD soil it was significantly lower when compared with Br, Coll, It4, Lit, Luv, Por, Pra2,, Pz, Sta, UAB, Vall, Vil (**Fig. 4**). In addition, offspring in Cam soil was significantly lower to that in OECD soil. All the soils accomplished the validity requirements of ISO 11267 [32], as the number of juveniles was above 100 and the variation coefficient was below 30%, with the exception of some soils with a slightly higher coefficient: Por (32%), Riu (32%), Sta (41%), and Vall (45%). Given the main aim of this study, we considered the reproduction in these soils as sufficient.

Some of the explanatory variables (soil properties) presented high correlation: N and C (0.97), and clay and WHC_{max} (0.79). Then, according to the progressive removal of the variable with higher VIF value, a set of seven variables was selected (all with VIF values below 10): pH, Fine sand, silt, clay, C, moisture, and %WHC. The GLM of the rate of individuals in the test soil with respect to the soil properties rejected pH and clay content as explanatory variables for the final model, with an AIC=10207 (AIC = 10448 in the full model) (**Table 3**). Hence, the final model was expressed as Juveniles = e^[(7.292047)-(0.017051•Finesand)-(0.010458•Silt)-(0.579443•N)-(0.031067•CEC)-(0.010796•%WHC)+(0.050383•Moisture)] (Do the asteriks represent product dots? (•) . The R^2 of the model indicated that 45% of the variance of the reproduction was able to be explained by the model, indicating an acceptable predictability of the model.

DISCUSSION

Representativeness of the selected soils

The soils studied covered a wide range of soil properties. According to the PCA factor scores of their properties, the lack of separated soil clusters indicates a gradual distribution of some of the soil properties, showing their suitability for the purposes of this study. Only Sta and OECD soils appeared clearly separated due their contrasting properties compared to the remaining soils (high organic carbon and total N contents and elevated fine sand content, respectively). Thus, the selected soils represented the diversity of natural soils that might be required for an environmental risk assessment of contaminated soils.

Influence of soil properties on avoidance behavior

Avoidance tests are based on the fact that organisms possess chemoreceptors, highly sensitive to substances present in the environment [21, 50]. It is an ecologically relevant endpoint, since avoidance might be a key strategy for soil organisms facing pollution. More precisely, avoidance can allow soil organisms to take refuge in relatively clean soil pockets at contaminated sites, preserving donor populations for recolonization and recovery [47]. This is specifically important for soil organisms due to their limited dispersal and colonization capabilities [51, 52]. By maintaining soil invertebrate biodiversity, this endpoint reflects the quality of soil as a habitat. Therefore, it can be used as indicator in risk assessment studies [8, 53, 54].

However, several limitations have been reported about the usage of avoidance tests. First, it has been shown that some neurotoxic pollutants might inhibit the individuals' locomotion, something that could distort the avoidance patterns, as already observed in earthworms [6, 55, 56], collembolans [57], and suggested for isopods [20]. Second, these methods are only applicable to the pollutants perceived via chemoreceptors [7]. Third, avoidance response to pollutants is also highly dependent on the species [4, 58, 59], according to their different capacities of chemoreception and locomotion. Finally, it has been suggested that in practice, and especially when using field soils, differences in soil properties between the reference soil and the tested soil may influence the avoidance response, meaning pollution cannot be identified [30]. For this reason, avoidance tests, when used as screening tool in site specific risk assessment, should only be considered if the similarity between test and reference soil is ensured, or if afterwards corrections can be done to compensate different soil properties. However, not much is known about soil preferences of ecotoxicological test species, pointing out the need of more information.

Avoidance patterns observed for *F. candida* appeared in most of the soil combinations tested (74%), suggesting a high influence of the type of soil tested on this response. However, the GLM analysis almost failed in identifying soil properties influencing the avoidance response of this species. More precisely, the model derived suggest that individuals might avoid the test soil when the moisture was lower than that in the control soil, but the model only accounted for 16% of the variance in their response. In addition, the contribution of soil moisture is not significant. This result seems to be consistent with the consideration of collembolans as particularly vulnerable to dry conditions [60] and the insensitivity of this species to soil properties [4, 28, 61], so other unmeasured variables (e.g., microorganisms) should be a possible explanation to the avoidance behavior observed.

The inability of the model to explain the avoidance behavior might be related to the limited set of comparisons carried out, always within regions. However, this outcome might also be explained by the influence of other soil parameters not assessed. As an example, differences in the microbial populations present might be important for the avoidance behavior of this species, since like other collembolans, it grazes on various groups of microorganisms [62, 63]. Kaneda and Kaneko [64] also reported that *F. candida* body growth increases with bacterial activity**.** Differences in microbial biomass might also explain why the OECD soil is the most frequently soil avoided by *F. candida* (75% of the comparisons). Despite of the fact that even OECD soil can sustain microbial populations [65-66], (where is the closed parens?) their levels should be low since the avoidance assay only lasted 48 h, a period too short for the recovery of microbial communities in this soil. However, due to the lack of microbial data in our study no further evaluation of this point is possible.

Influence of soil properties on reproduction

The results reported here indicate that nearly all the natural soils tested are suitable for reproduction tests as they fulfil the validity criteria established in the ISO (2010). The exception was Pra soil, where all the individuals died. This is consistent with the high mortality rates also observed in the avoidance tests with this soil (above the 20% maximum mortality set as validity criteria). This result indicates the usefulness of avoidance tests as a fast screening method for evaluating the habitat function of soils [12]. The unexplained high mortality in this soil remains unclear. However, it might be related to agricultural practices not reported by the land owner, like nitrogen fertilization, shown to impact soil fauna densities in the short-term [67], as well as pesticide application.

In the reproduction tests with *F. candida* clearly contrasting numbers of juveniles were observed in the different soils (ranging from 242 to 1210 juveniles), which, besides the demonstrated individual variability in fertility in this species [68], where is your ending paren? should unequivocally be attributed to soil properties. It is also remarkable that reproduction in natural soils is at least equal but often higher than in OECD soil used in our study. The influence of soil parameters on the reproduction outcome of soil organisms has been widely reported in the literature, but less ~~it~~ is known about the effects on the collembolan *F. candida* besides the scarce published studies [3, 13-16, 28]. According to our results, reproduction of this species was significantly lower in the more fine-textured soils (with higher silt and fine sand content and with higher CEC values). ~~On the other hand~~ However, reproduction was positively and significantly affected by soil moisture. In addition, there is also a negative influence of total nitrogen content and moisture (measured as %WHC), despite of the fact in both cases their significance was lower than the remainding properties. The negative influence of %WHC on reproduction, contradicting the clear positive effect of soil moisture, might be a mathematical artefact rather than a real influence, since moisture and %WHC should be correlated. In fact, both properties were positively related in the set of soils tested, but the correlation was marginally not significant (Pearson, p=0.059). The positive influence of moisture on reproduction and avoidance found in our study confirms data from literature, indicating that reproduction in OECD soil decreases below 50% of the WHC and even can cease below 30% of the WHC [25, 26]. ~~On the other hand~~ However, in very wet soils, above 75% of the WHC, a strong decrease of reproduction has also been observed ([24]. The latter fact could not been confirmed here since our soils were moistened to ~~around~~ approximately 30 to 60% of the WHC.

The lower reproduction in soils with increasing content of fine particles is in accordance with [13], which results suggest a positive, though not significant, association among the number of juveniles produced and the sand content. This trend might be related with a low performance in fine textured soils, more hardly colonized and less utilizable by these species due to its relatively big size.

A significant influence of increasing levels of total nitrogen on an decrease of the number of juveniles produced was also observed, which could be related with the noxious effect of some nitrogen-derived compounds (in particular ammonia) released during the tests. This chemical is known to decrease field populations of soil fauna [67, 69], and to impact directly the survival of *F. candida* [70]. The combination of favourable conditions (optimum moisture and temperature) for the degradation such compounds during the tests, together with the limited aeration in the test containers (aerated twice a week), are likely to magnify the release and noxious effects of the nitrogenated endproducts on collembolans.

Different studies have suggested a negative influence of pH on the number of offspring [23, 25, 27], something that we did not find in our study. These studies have indicated that reproduction in this species is maximum at 5.5, and reduced over and below this value. Despite most of our soils presented pH over 5, we failed in finding a significant influence of pH in reproduction, in agreement with [13].

CONCLUSIONS

The influence of soil properties on the avoidance behavior of this species seem to be low, being soil moisture the only influential property, with lower avoidance and higher reproduction the higher the moisture level. However, this was only significant for reproduction and marginally not significant for avoidance. In addition, reproduction was also shown to be the lower the more fine-textured and the richer in nitrogen is the soil.

However, the outcomes in most of the soils accomplished the validity criteria, agreeing with the consideration of this species as relatively insensitive to soil properties and hence highly suitable to be used in ecotoxicological tests with natural soils, especially in avoidance tests. In addition, our results also point the need of accuracy in the adjustment of soil moisture to ensure the quality of avoidance and reproduction test results when this species is used.

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Figure Legends

Figure 1. Distribution of the soils according to the principal components analysis (PCA) factor scores for the three main axes summarizing their properties. See Table 1 for soil abbreviations. Refer to Table 1 for Soil site abbreviations. OECD = Organization for Economic Cooperation and Deveopment.

Figure 2. Dual-control tests results of *Folsomia candida*, expressed in percent of the individuals present in each side (consisting on the same soil in both sides). The equal distribution of individuals in both sides could not be rejected for any of the soils according to the two-tailed Fisher's exact tests carried out. Bars indicate standard deviation. See Table 1 for soil abbreviations. Refer to Table 1 for soil site abbreviations. OECD = Organization for Economic Cooperation and Deveopment.

Figure 3. Distribution of *Folsomia candida* in soil combinations involving artificial soil as set by the Organization for Economic Cooperation and Development (OECD) (upper figure) or only natural soils (lower figure). Bars represent the percent of individuals in control soil (left bar) compared to that in the test soil (right bar). The unequal distribution of collembolans (one-tailed Fisher's exact test), is indicated by the asterisk. Avoidance response to the test soil is shown by lower percent of individuals in this soil (right bar), while a preference for the test soil is found if the opposite occur. See Table 1 for soil abbreviations. Refer to Table 1 for soil site abbreviations.

Figure 4. Reproduction of *Folsomia candida* in different soils, expressed as number of juveniles. Asterisks indicate significant differences with respect to the outcomes in the artificial soil prepared as set by the Organization for Economic Cooperation and Development (OECD)

(Fisher's least significant difference test, $p<0.05$). Bars indicate standard deviation. See Table 1 for soil abbreviations. Refer to Table 1 for soil site abbreviations.

Table 1. Properties of the soils studied, all coming from three European Mediterranean regions: Alentejo (Portugal), Catalonia (Spain) and Liguria (Italy). *Coarse sand* = 2 - 0.2 mm; *Fine sand* = 0.2 - 0.02 mm; *Silt* = 0.02 – 0.002 mm; *Clay* = < 2 µm; *C* = organic carbon; *N* = total nitrogen; *CEC* = cationic exchange capacity; *MaxWHC* = maximum water holding capacity; *Moisture1* = soil moisture in avoidance tests; *Moisture2* = soil moisture in reproduction tests; %*WHC1* = soil moisture in avoidance tests expressed as percent of the maximum WHC; %*WHC2* = soil moisture in reproduction tests expressed as percent of the maximum WHC. All the values are referred to the dry matter. The data gaps in Moisture1 and %WHC1 are due to the fact that these soils were exclusively used in reproduction tests (Cam, Coll, Pra2, Sta, UAB, Vall and Vil).

Soil	Site	Soil use	pH	Coarse sand	Fine sand	Silt	Clay	C	N	CEC	WHCmax	Moisture1	Moisture2	%WHC1	%WHC2	Cd	Cr	Cu	Ni	Pb	Zn
			water, 1:5	%	%	%	%	%	%	cmol kg^{-1}	%	%	%	%	%	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Br	Beja, Portugal	Agricultural	7.6	10.0	17.9	23.6	48.4	1.45	0.11	26.8	61.1	24.6	19.9	40.3	32.6	<5.6	67	46	66	18	55
Cam	Campdàsens, Spain	Chaparral	7.9	13.2	18.8	37.9	30.1	4.85	0.30	22.3	44.9	-	16.1	-	35.8	0.2	39	23	19	23	94
Coll	Collsacreu, Spain	Woodland	4.9	51.1	21.6	17.6	9.70	2.19	0.11	9.20	33.9	-	13.6	-	40.1	<0.1	10	8.0	<10	17	62
Gan	Gandesa, Spain	Vineyard	8.3	1.52	74.4	12.0	12.0	0.35	0.04	6.00	37.6	15.1	15.0	40.2	39.8	<0.1	16	20	32	11	32
Gra	La Granadella, Spain	Olive field	8.2	2.08	25.7	48.5	23.7	0.99	0.11	14.2	49.8	16.3	16.3	32.7	32.6	0.2	19	26	28	10	42
It2	Ventimiglia, Italy	Agricultural	7.7	23.6	15.7	44.4	16.2	2.78	0.25	18.6	39.8	20.8	21.4	52.1	53.7	0.6	61	172	48	59	170
It3	Ventimiglia, Italy	Fallow	7.7	23.1	28.1	36.3	12.4	1.62	0.16	18.4	43.3	21.7	20.5	50.1	47.3	<0.1	71	48	54	18	76
It4	Ventimiglia, Italy	Fallow	7.8	20.2	29.0	34.7	16.0	1.62	0.13	18.8	47.4	21.5	23.4	45.3	49.3	<0.1	67	34	54	21	76
Lit	Mértola, Portugal	Fallow	5.2	41.9	24.8	21.5	11.7	2.44	0.16	8.64	42.4	19.6	19.7	46.3	46.4	<5.6	21	40	48	16	67
Luv	Mértola, Portugal	Pasture	5.5	29.8	38.2	20.3	11.3	1.16	0.08	9.92	32	17.0	16.5	53.0	51.6	<5.6	24	23	28	19	54
Por	Porrera, Spain	Vineyard	6.9	46.2	21.4	20.4	11.9	2.49	0.22	18.6	38.8	16.4	16.5	42.3	42.6	0.2	67	92	46	147	420
Pra	Prades, Spain	Grainfield	5.1	42.4	35.0	12.1	10.5	1.28	0.12	11.2	39.4	15.3	15.7	38.8	39.9	<0.1	13	38	5.5	40	86
Pra2	Prades, Spain	Grassland	6.5	60.8	19.5	16.5	3.20	1.12	0.10	7.30	28.1	-	11.6	-	41.3	0.9	11	77	<10	48	204
Pz	Vilafranca de Xira, Portugal	Pasture	5.3	69.8	21.3	5.70	3.19	1.28	0.07	4.04	30.7	10.0	19.4	32.7	63.1	<5.6	<16	<15	<28	7.0	6.0
Riu	Riudellots, Spain	Grainfield	7.3	23.6	34.9	13.8	27.6	1.10	0.13	14.9	45	16.3	16.9	36.2	37.5	<0.1	22	26	18	19	64
Sta	Sta Fe, Spain	Woodland	5.7	69.3	10.9	8.70	11.1	6.94	0.53	17.7	50.7	-	20.3	-	40.0	<0.1	13	11	<10	41	145
UAB	UAB campus, Spain	Grainfield	8.3	25.8	13.9	44.9	18.7	2.63	0.18	13.9	49.5	-	25.0	-	50.5	<0.1	25	121	19	35	104
Vall	Vallgorguina, Spain	Woodland	6.4	72.4	12.1	8.30	7.20	1.26	0.08	9.20	29.9	-	12.0	-	40.1	<0.1	9.0	16	<10	14	85
Vil	Vilanova de Prades, Spain	Grassland	8.4	16.5	9.0	54.6	19.9	0.65	0.05	8.1	48.0	-	19.9	-	41.5	<0.1	28	6.0	13	21	43
OECD	Artificial soil	-	7.0	9.74	76.9	2.70	10.7	3.36	0.03	7.04	63.1	27.3	22.0	43.3	34.9	0.1	8	20	3	10	15

Table 2. Model fit of the logistic regression model and numerical output for the avoidance of collembolans.

	Estimate	*Standard error*	*t-value*	*p-value*
Intercept	-0.8422	0.179	-4.705	<0.01
Q_moisture	0.2287	0.1278	1.789	0.0736

Null deviance: 14.78 on 131 degrees of freedom

Residual deviance: 12.32 on 131 degrees of freedom. AIC: 173.2

Table 3. Model fit of the Poisson regression model and numerical output for the reproduction of collembolans.

	Estimate	*Standard error*	*t-value*	*p-value*
Intercept	7.292	0.207	35.25	<0.001
Fine sand	-0.017	0.002	-8.252	<0.001
Silt	-0.017	0.002	-4.471	<0.001
N	-0.579	0.273	-2.116	0.036
CEC	-0.031	0.006	-4.852	<0.001
%WHC	-0.011	0.005	-2.138	0.034
Moisture	0.050	0.012	4.253	<0.001

Null deviance: 14869.8 on 144 degrees of freedom

Residual deviance: 8171.4 on 144 degrees of freedom. AIC: 10207

Figure 1

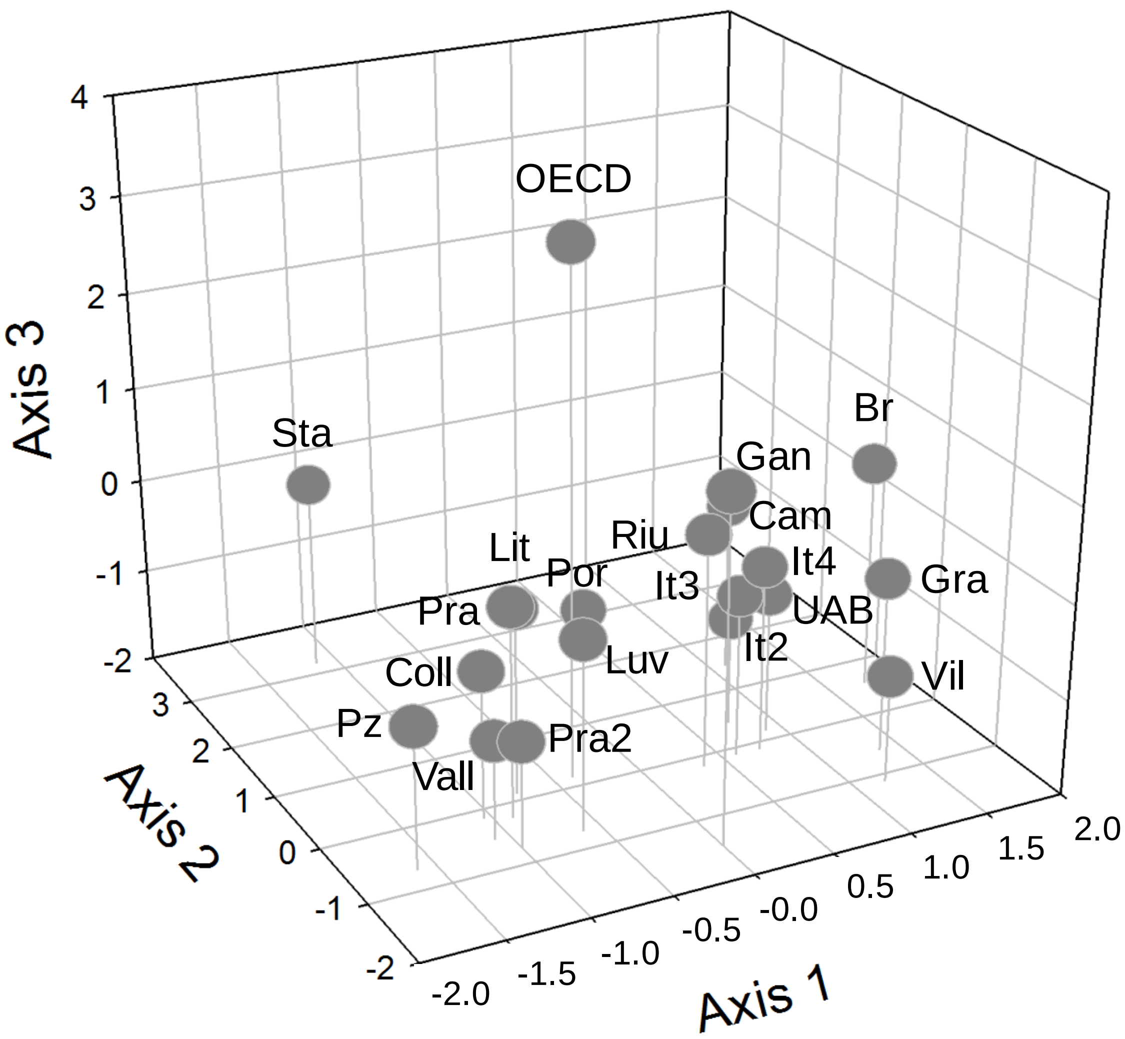
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Figure 2

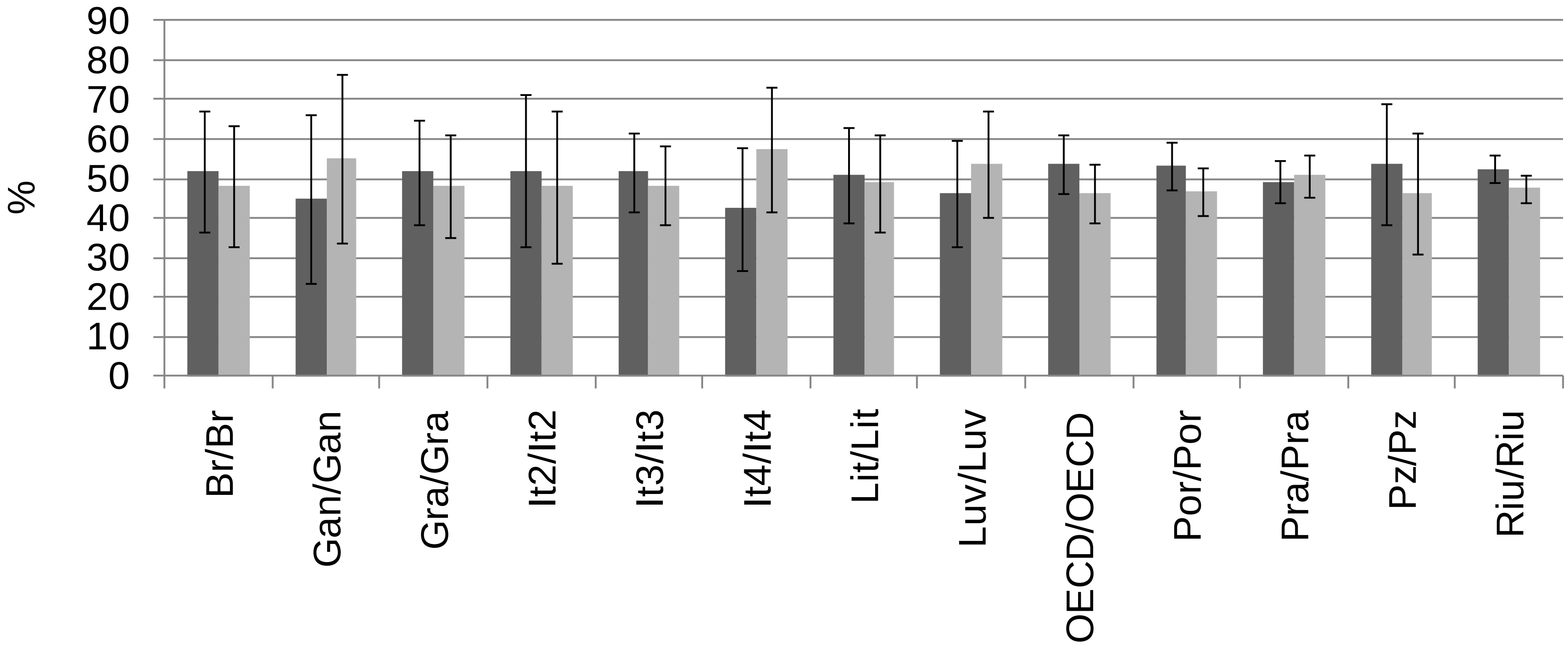

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Figure 3

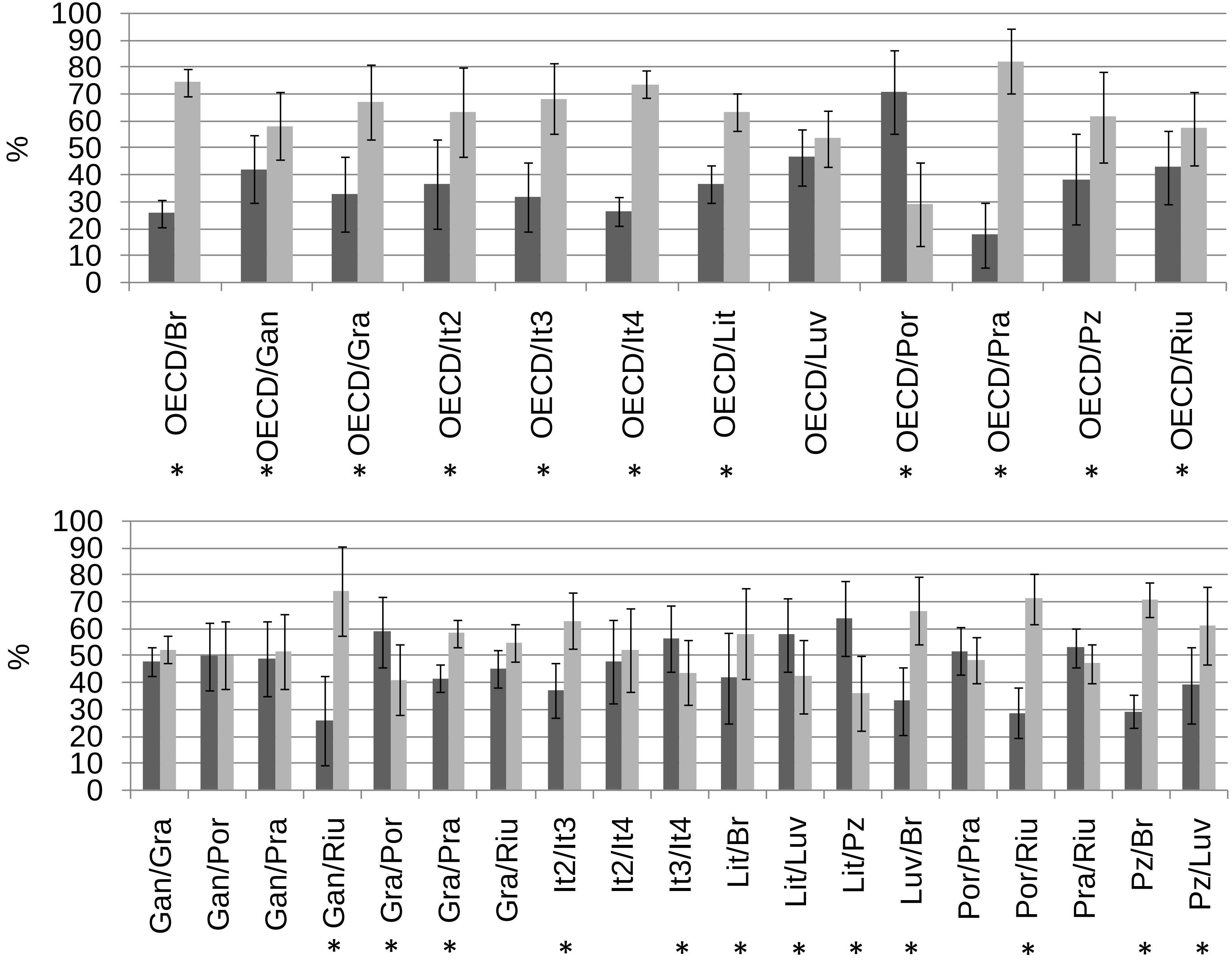

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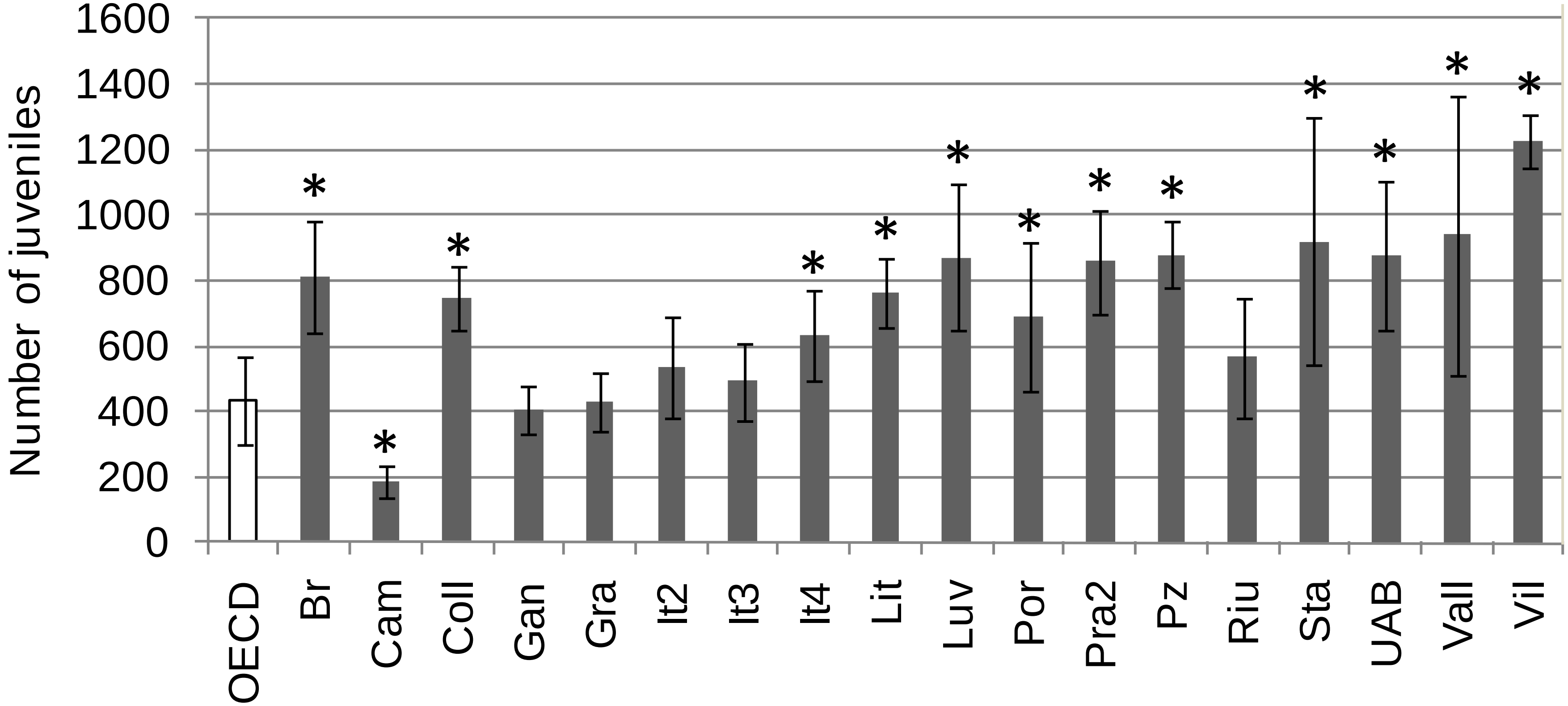
Figure 4



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