

E X T E R N A L M E M O R A N D U M

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SUBJECT: Variability in enzyme activity results

One of the primary reasons for conducting the follow-up bioavailability study was to assess the possible impact of the differential enzyme induction observed in the pilot study on the estimation of relative bioavailability of dioxin/furan compounds in soil. In the process of reviewing the enzyme activity data, some differences were noted and explored in more detail.

The EROD results for the follow-up study were noticeably lower overall than those observed in rats during the pilot study, even when the differing dose levels were taken into account. As can be seen in Figure 1, when EROD results are plotted against hepatic TEQ, the group mean EROD results for the follow-up study (excluding controls) range from 42 to 143 pmol/mg/min, with an average of 88 pmol/mg/min, while EROD results from rats in the pilot study range from 63 to 486 pmol/mg/min, with an average of 222 pmol/mg/min for rats in the pilot study. The MROD activity results were also lower in the follow-up study than those for rats in the pilot study (Figure 2).

To investigate the potential source of this variability in the EROD results, more than half (16 of 30) of the rat liver microsome samples from the follow-up study were re-analyzed in the same laboratory, and were also analyzed at a second outside laboratory, in which rat liver microsomes from the pilot study had been analyzed in 2004. The reason for the inter-laboratory comparison was that a new multiwell fluorescence plate reader (Fluoroskan) had been used to analyze the follow-up samples, and this instrument used different filter sets to measure protein and resorufin. The instrument used with the 2004 samples used excitation and emission monochrometers to set the appropriate wavelengths (Molecular Designs). Thus, the two primary sources of variability that were investigated in these additional analyses included reagent purity and calibration of the fluorescence microtiter plate reader that was used in the EROD assays. Reagents that were evaluated included assay buffers, the working resorufin stock solutions, and the NADPH stock. The fluorescence reader comparisons focused on measurements of protein and resorufin. For protein analysis, microsomal proteins were measured with two methods—the Bradford method and the fluoroscamine method, in which samples on the same microwell plate were measured on both instruments. For resorufin, EROD bioassay was conducted with rat liver microsomes, and the multiwell plate was analyzed on both

fluorometers. When the new reagents were used in the EROD assays, the measured activity was similar to that observed in the initial follow-up study results, with activity levels typically being less than 10% different from that measured in the original analysis (Figure 3). This result indicates that reagent purity, and resorufin stock solution purity in particular, were not factors in the observed differences. When protein concentrations for a selected number of microsomal samples were measured by the Bradford method and by the fluoroscamine method using the two different fluorometers, the resulting protein concentrations were similar, with differences being typically less than 10% among all three measurements. Finally, when resorufin concentrations measured on both fluorometers were compared, no significant differences were observed in absolute resorufin concentration measured for each sample, nor were there any significant differences between measured EROD activities between the two fluorometers. These results indicate that the differences between the pilot study and the follow-up study results were not a function of differences in instrumentation or reagent purity. Thus, the initial results appear valid, and were used for the analysis in the report.

To further explore this variability, six rat liver samples from the pilot bioavailability study were re-analyzed. The results obtained were lower than those reported in the pilot study, which was not surprising and may indicate sample degradation. In general, though, these results were on the high end of the range when compared to what was observed in the follow-up study (Figure 4).

To place this variability in a wider context, the results from the above-described studies were plotted along with the results of the background study done in 2003 and three dioxin/furan compounds that were evaluated by the National Toxicology Program (NTP) (Figure 5). The EROD results from the NTP study were significantly higher than were observed in any of the studies associated with the Midland site. This comparison demonstrates that the variations observed between the pilot and follow-up study, while important in terms of calculating relative bioavailability for the Midland studies, are not all that large compared to the universe of available EROD data.

In conclusion, it is clear that the potential exists for significant variability in enzyme activity results, apparently due to assay conditions or variations in animal characteristics, even when animals are dosed with the same material containing the same congener concentrations. Thus, simultaneous reference groups should be used for all future studies. Based on the replication of the follow-up study EROD results, the initial results appear to be valid and were used in the analysis.

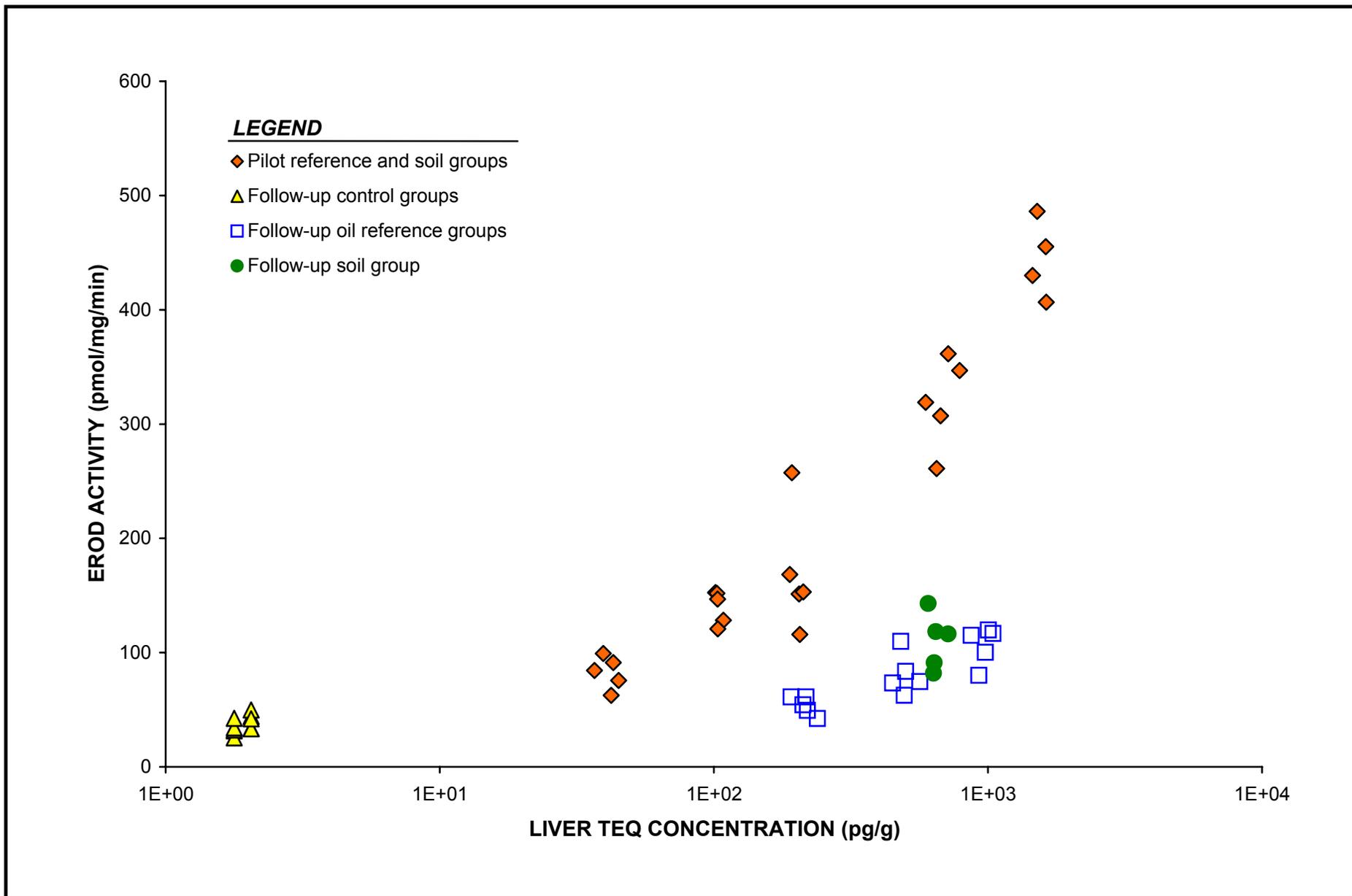


Figure 1. Comparison of EROD activity results from the pilot and follow-up studies

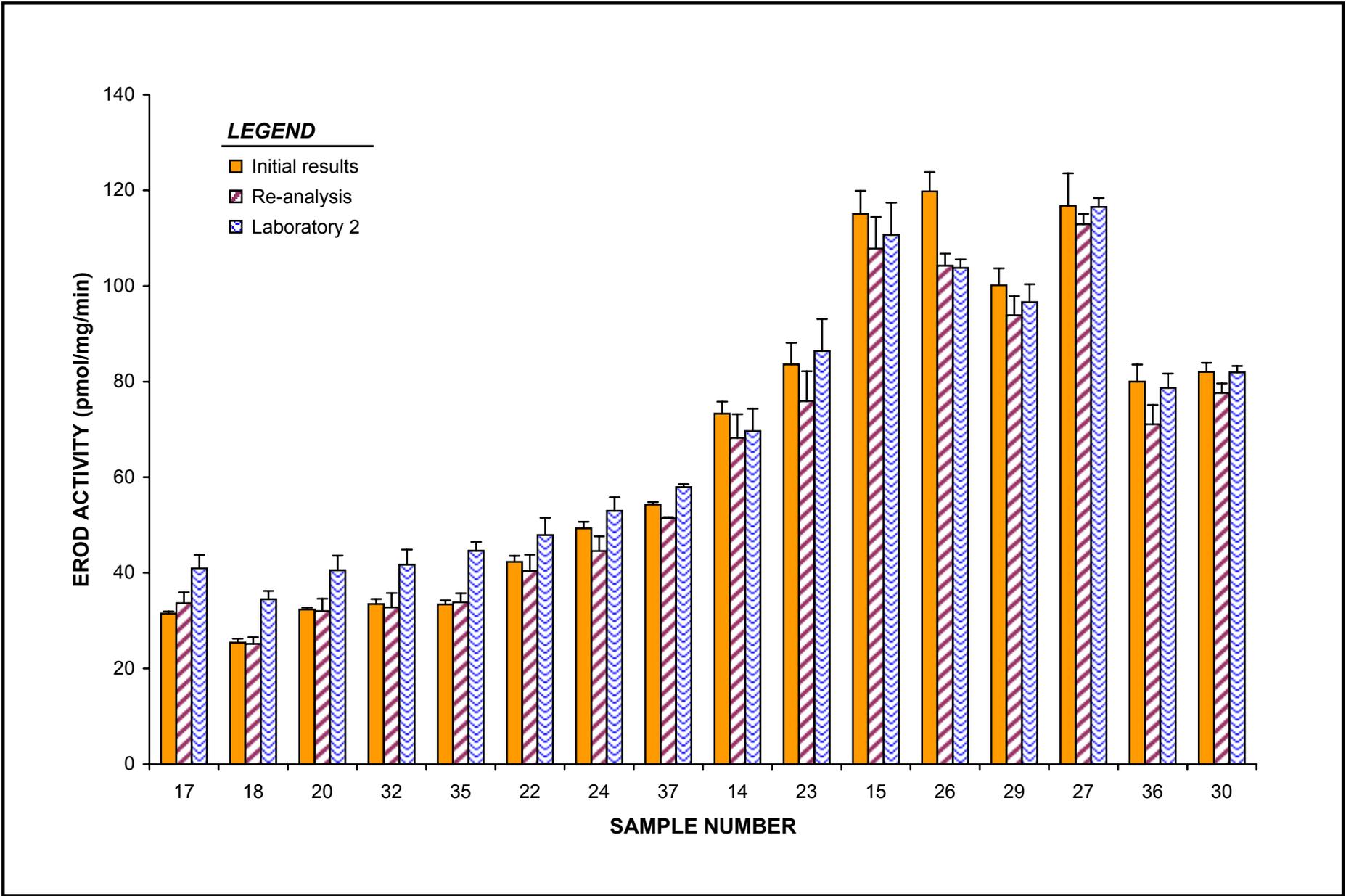


Figure 3. Confirmation of EROD results in the follow-up rat study

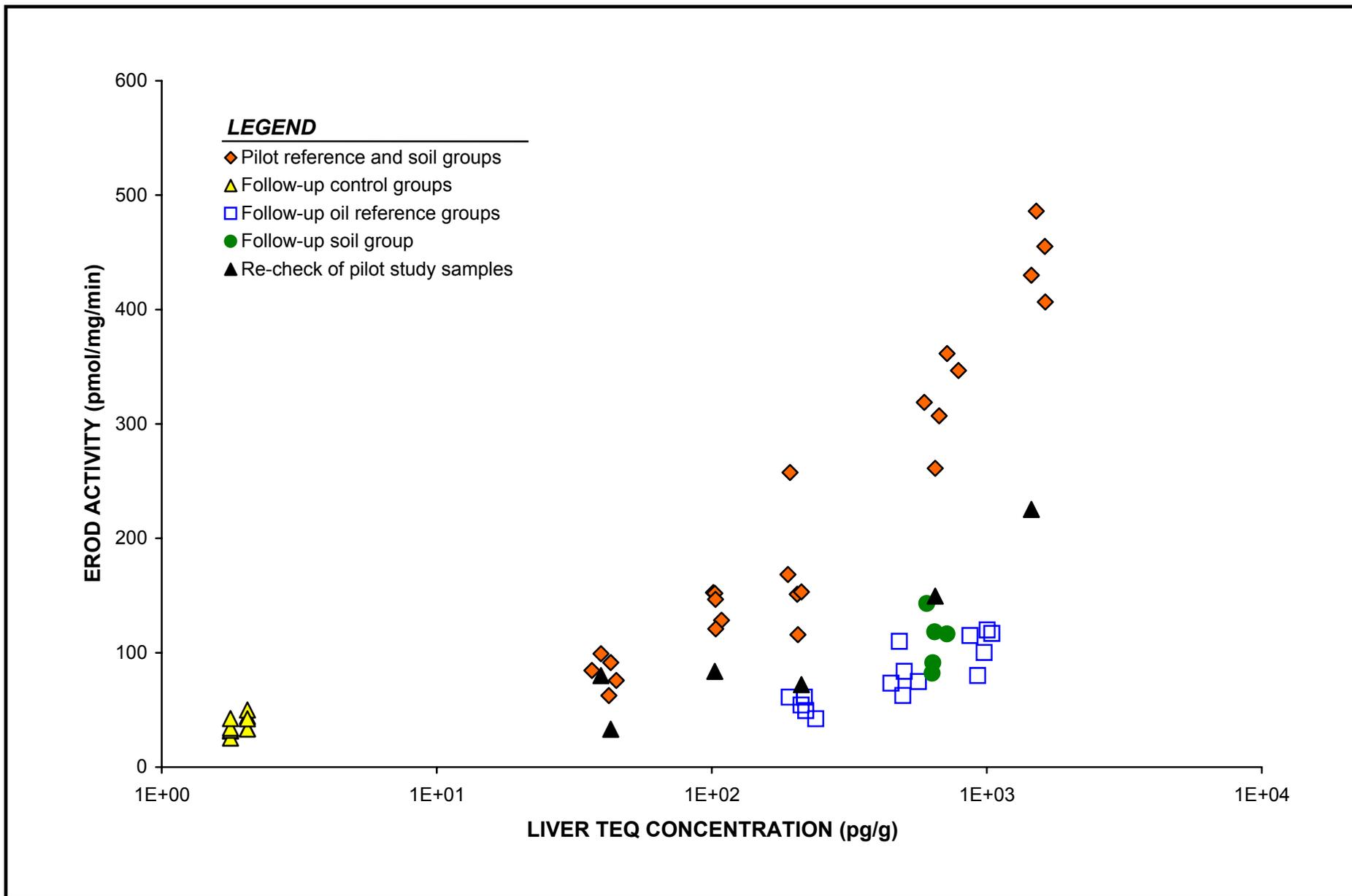


Figure 4. Comparison of EROD activity results from the pilot and follow-up studies, plus a re-check of pilot study samples

