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Sakrapport

Adjustments for confounders in pooled samples

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Naturhistoriska riksmuseet

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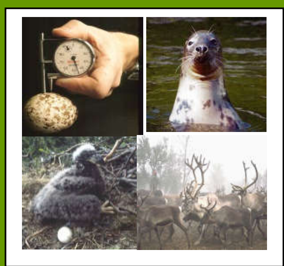

Adjustments for confounders in pooled samples

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Confounding factors like age and/or fat content influence the measured concentration of contaminants in biological samples. If the average age or fat content varies over time or between sites, the variance in the measured concentrations will tend to increase, and decrease the statistical power to detect temporal trends or differences between sites. It is a well studied fact that e.g. mercury (Hg) and dioxins accumulates with age, and that cadmium (Cd) concentration (dry weight, dw) in cod liver are affected by the liver fat content. Therefore a comparison of e.g. Hg concentration in fish muscle over time should present mercury concentrations adjusted to the same age (if age is not determined a proxy for age like length could be used). When pooled samples are used, each pool may contain specimen with varying age and fat content. The objective with this report is to compare the effect of adjusting for confounders in individual and pooled samples respectively.

As real examples Cd in cod liver and Hg in perch muscle (see Appendix) were studied. It is well known that Cd (dw) correlates with liver fat content in cod (e.g. Grimås *et al.*, 1985). Also length and age were tested using multiple regression, but did not affect the Cd-concentration significantly when tested together with liver fat%. Three scenarios are presented below (Fig.1-3).

In the first scenario, Fig.1, the individual measurements are considered independent and analyzed individually. This will result in the most sensitive design to detect trends; after adjustment for liver fat content we expect to be able to detect a trend of 4.4% a year within a period of 10 years with a statistical power of 80%. However, independence of the samples can be questioned if all cod specimens are caught at the same time and at the same spot in a year (temporal and spatial autocorrelation).

Legend to the Figures

The first panel show unadjusted data (light blue dots).

n(tot) = total number of analysed individual specimens, **n(yrs)** = number of years

slope = yearly change in percent with a 95% confidence interval

CV(lr) = residual variance around the regression line expressed as a Coefficient of Variation (%), followed by the estimated yearly change, possible to detect at a statistical power of 80% for the current time series (i.e. for the example below, during a study period of 28 years), followed by the number of years required to monitor, to detect a 5% yearly trend.

LDT = Lowest Detectable Trend expected to be detected with a statistical power of 80% during a monitoring period of 10 years.

r^2 = Coefficient of Determination for the log-linear regression analysis followed by a p-value

The second panel shows the outcome from the multiple regression analysis. The horizontal bar chart shows the size of the standardized beta-coefficients, i.e. the relative importance of the variables in the model. The direction indicates the sign of the beta-coefficients, bars to the left indicate negative coefficients, to the right positive coefficients. The colours of the bars indicate the p-values of the beta-coefficients, unfilled = $p > 0.05$, pink= $p< 0.05$, red = $p<0.01$, purple = $p <0.001$.

The third panel shows the observed values adjusted for the variables in the multiple regression model ***except for year*** (red dots), i.e. the adjusted values is estimated to values that we would expect if the confounding variables were constant (at the recorded mean value) and assuming that the multiple regression model is correct. The statics reported in the upper left of the figure contains the same type of information as described for the left panel (see above).

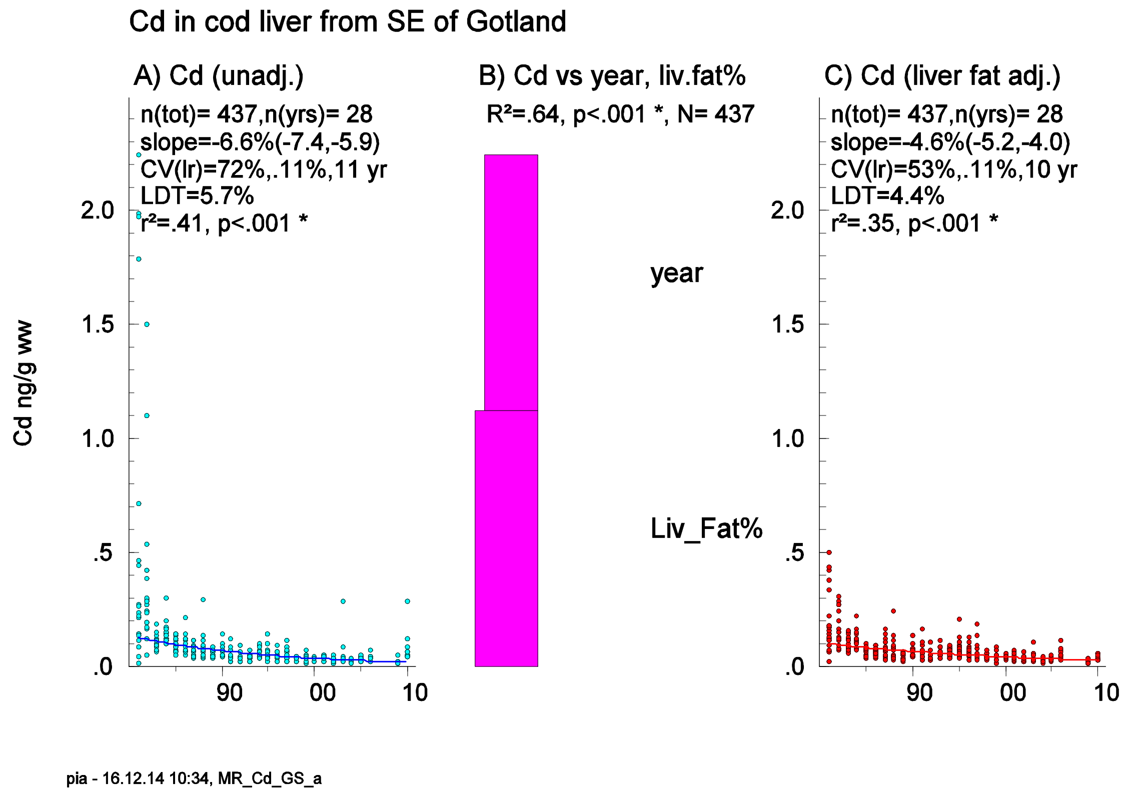

Figure 1. Temporal trend assessments based on individual specimens. **A)** Unadjusted Cd concentrations over time, in cod liver from SE Gotland. **B)** Multiple regression show that logged cadmium concentrations expressed on a dry weight basis is highly correlated with the fat content in cod liver ($p<0.001$). **C)** Cd-concentrations measured in individual cod specimens adjusted for liver lipid content. The Coefficient of variation (CV) decreases from 72% to 53% and the lowest detectable trend (LDT) expected to be found with an 80% power during a study period of 10 years improves from 5.7% to 4.4%.

The second scenario (Fig.2, below), describes a situation with individual analyses and temporal trend analysis on geometric means from 20 specimens annually, between 1981-1996, and 10 specimen from 1997-2006. This is the procedure that has been used in our monitoring reports, since the individual measurements cannot be considered fully independent within a year (the degree of independence between specimens within a year caught at the same site and time varies due to sampling strategy and species; e.g. cod specimens, maybe not all caught the same day in the same net, are probably more independent than herring specimens caught from the same school).

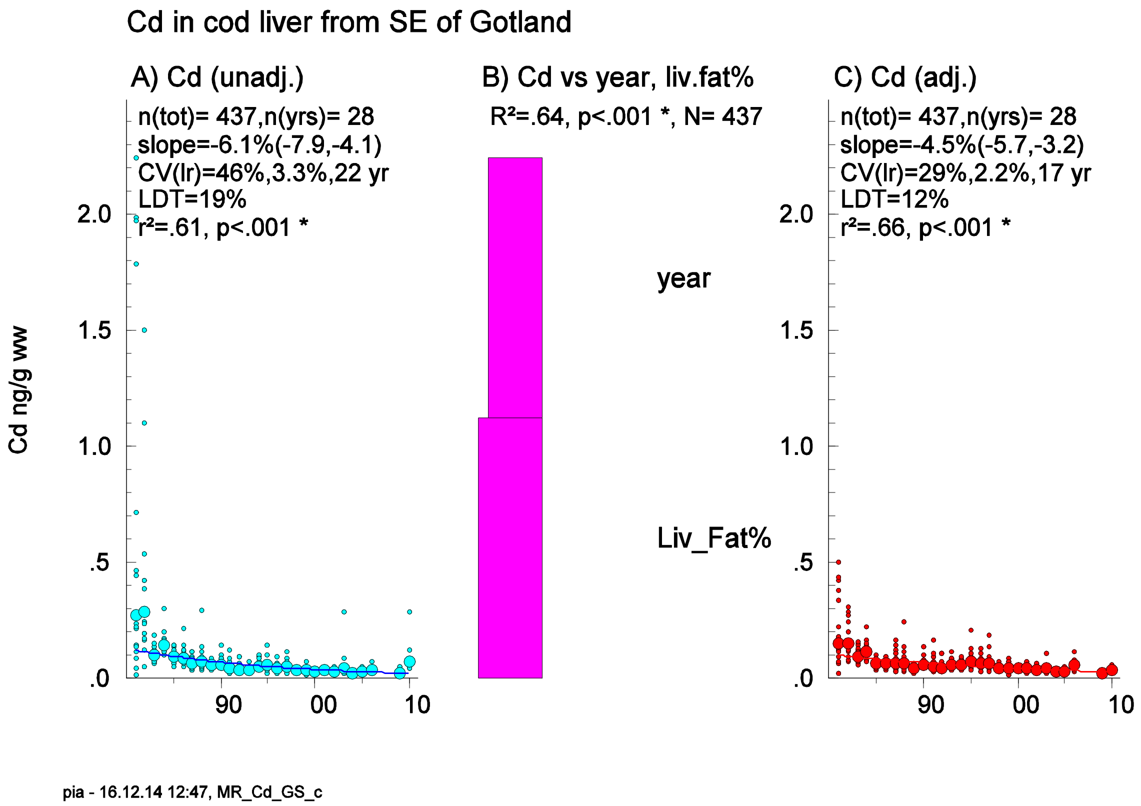

Figure 2. Temporal trend assessments based on yearly geometric mean values. **A)** Unadjusted yearly geometric mean values. **B)** Multiple regression show that logged cadmium concentrations (geometric mean values) expressed on a dry weight basis is highly correlated with the fat content in cod liver ($p<0.001$). **C)** Cd concentrations adjusted for liver fat% before the formation of yearly geometric mean values. The Coefficient of variation (CV) decreases from 46% to 29% and the lowest detectable trend (LDT) expected to be found with an 80% power during a study period of 10 years improves from 19% to 12%.

In the third scenario (Fig.3, below), pooled samples (simulated from arithmetic means) were used and the adjustment used mean values of the confounding factor. The gain in statistical power as a result of adjusting for varying fat content in the cod liver is substantial; lowest detectable trend decreases from 24 to 16% and the n of years required to detect a 5% annual trend with a 80% power, decreases from 25 to 20 years.

The regression of the simulated pools is not altogether fair compared to a real example of pooled samples since the error from the chemical analyses is somewhat underestimated (the simulated pools, based on several individual results will reduce both the variance contribution from individual biological factors and from the chemical analyses, whereas in real pooled samples the variance due to the chemical analyses is not reduced by several chemical analyses). However, this error is usually much smaller than the error due to the individual biological differences and should not affect the results for the comparisons in this study. Analyzing only one pool compared to 10 or 20 individual specimens reduces the cost for chemical analysis to a tenth or to one-twentieth of the original price however to a cost of reduced power. For unadjusted measurements the expected lowest trend to be detected increases (at a statistical power of 80% during a period of 10 years) from 19 to 24%, for the liver fat adjusted measurements from 12 to 16%. This can of course be compensated for by analyzing more pools (also, to some extent, by using pools with more individuals) (Bignert *et al.*, 2014).

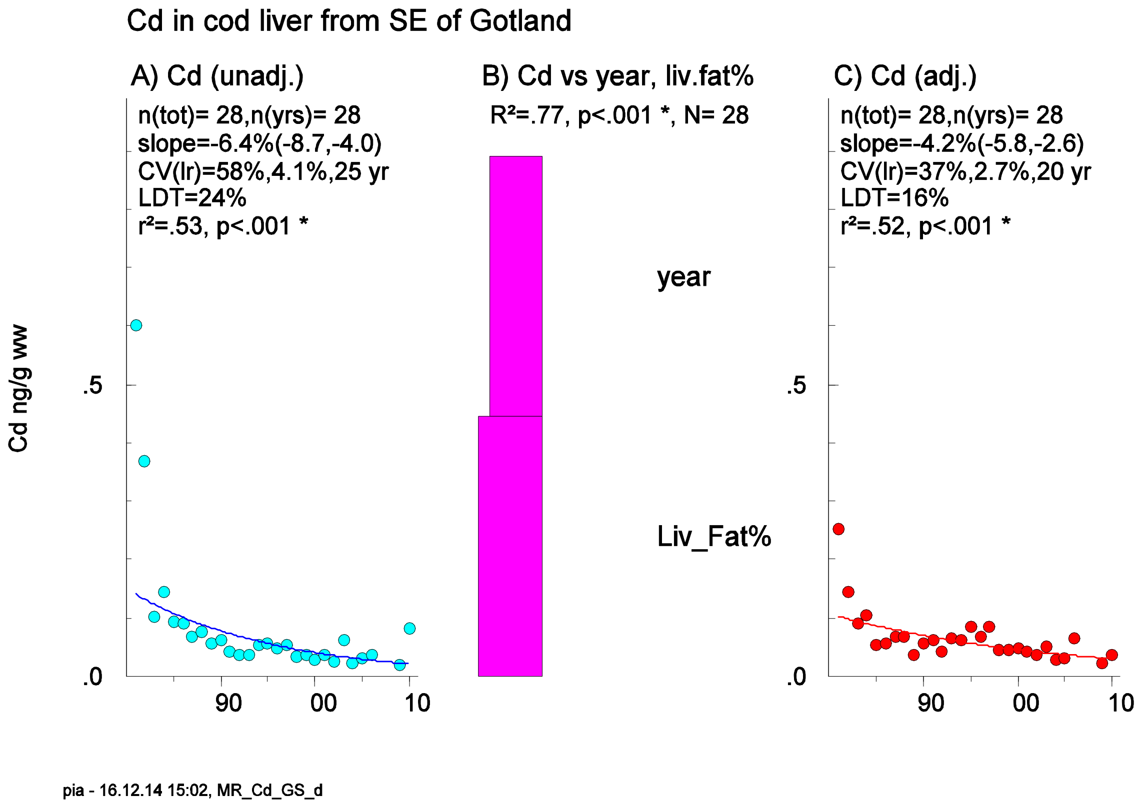

Figure 3. Temporal trend assessments based on pooled samples. **A)** Unadjusted pools. **B)** Multiple regression show that logged cadmium concentrations expressed on a dry weight basis is highly correlated with the fat content in cod liver ($p<0.001$) **C)** Pools adjusted for liver fat%. The Coefficient of variation (CV) decreases from 58% to 37% and the lowest detectable trend (LDT) expected to be found with an 80% power during a study period of 10 years improves from 24% to 16%.

Conclusion

Pooling is efficient in situations where 1) the analytical cost is much higher than the cost of sampling and preparing additional specimens 2) when the uncertainty due to the individual specimen (e.g. biological confounder like age, fat etc) is much higher than the uncertainty of the analytical precision and 3) where the individual specimens caught at the same spot at the same time of the year cannot be considered independent and thus is evaluated through yearly average values.

In situations where pooled samples are used, adjustments for mean values of confounders of pooled samples can often (but not always) substantially improve the power to detect trends. Since the costs for carrying out these adjustments is low, it should routinely be used also for pooled samples. It is thus essential that a search for relevant confounders is continued and that such confounders are included in the sampling protocols.

References

Bignert A., Eriksson U., Nyberg E., Miller A. & Danielsson S. 2014. Consequences of using pooled versus individual samples for designing sampling strategies. *Chemosphere, 94:177-182.*

Grimås U., Göthberg A., Notter M., Olsson M. & Reutergårdh L. 1985. Fat Amount - a Factor to Consider in Monitoring Studies of Heavy-Metals in Cod Liver. *Ambio, 14: 175-178.*

Appendix

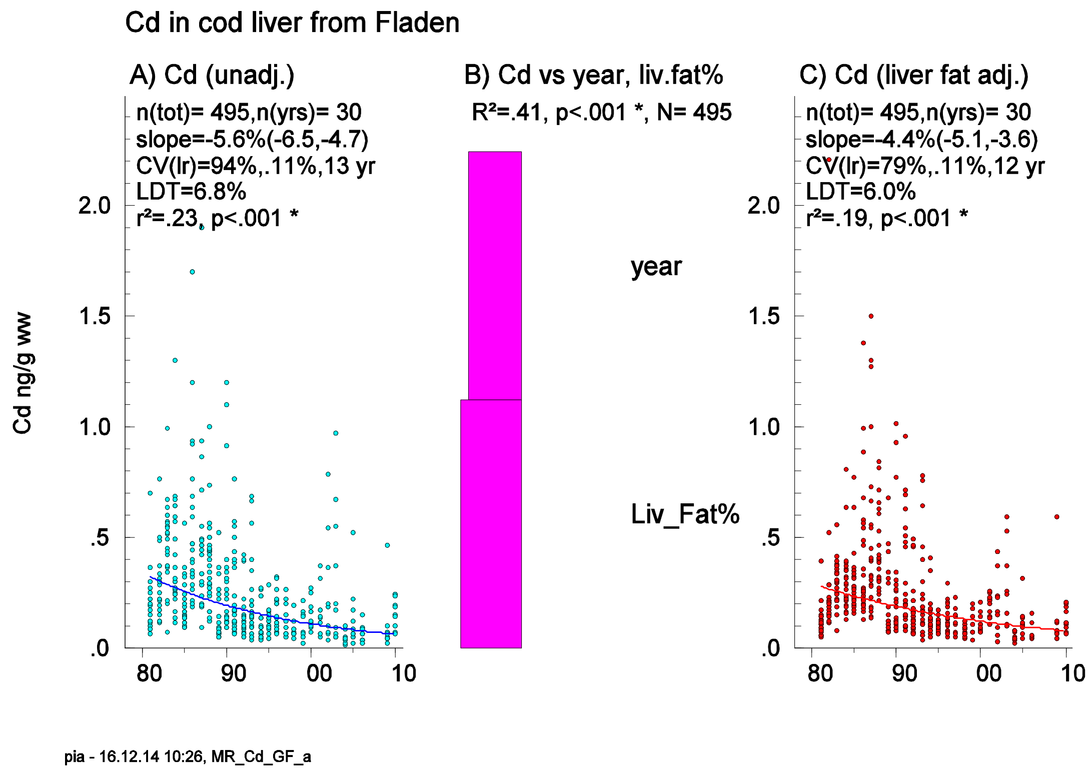

Figure S1. Temporal trend assessments based on individual specimens. **A)** Unadjusted Cd concentrations over time, in cod liver from Fladen. **B)** Multiple regression show that logged cadmium concentrations expressed on a dry weight basis is highly correlated with the fat content in cod liver ($p<0.001$). **C)** Cd-concentrations measured in individual cod specimens adjusted for liver lipid content. The Coefficient of variation (CV) decreases from 94% to 79% and the lowest detectable trend (LDT) expected to be found with an 80% power during a study period of 10 years improves from 6.8% to 6.0%. The log-linear trend fitted to the data is probably not optimal. Visual inspection of the observed data indicates an initial increase during the early 80-ies followed by a log-linear decrease starting in the mid 80-ies. This will lead to an increased between-year variation also after adjustment. Still the overall variance decreased after adjustment.

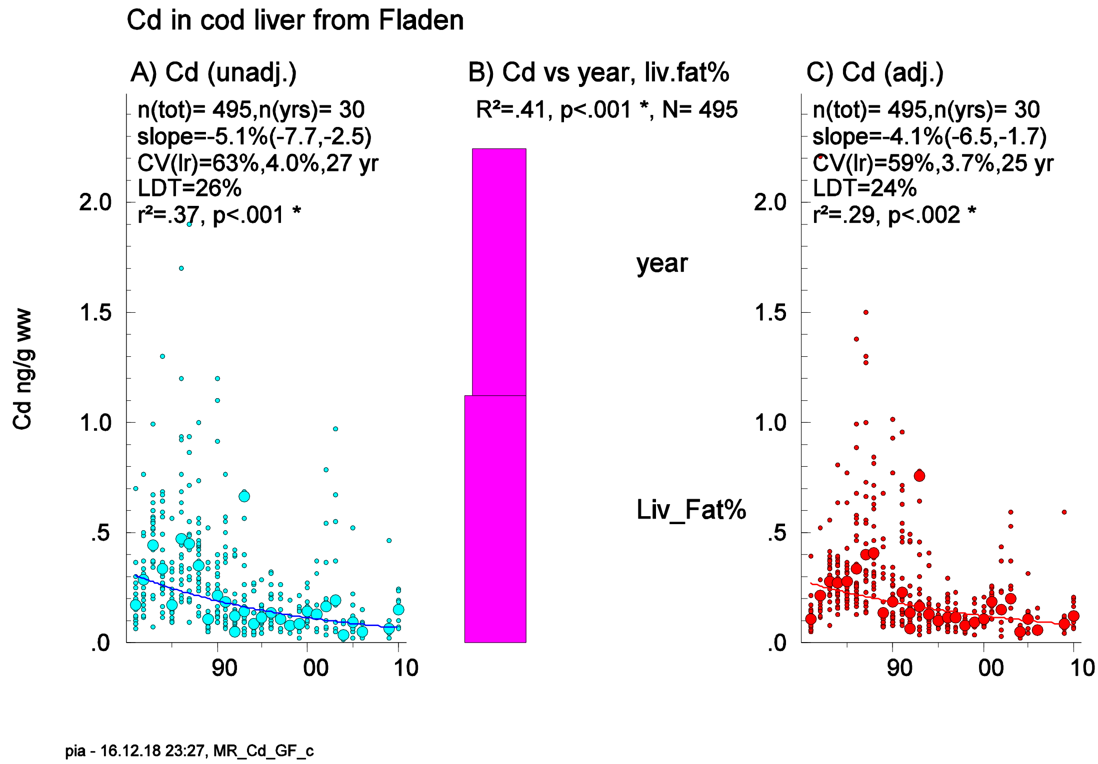

Figure S2. Temporal trend assessments based on yearly geometric mean values. **A)** Unadjusted yearly geometric mean values. **B)** Multiple regression show that logged cadmium concentrations (geometric mean values) expressed on a dry weight basis is highly correlated with the fat content in cod liver (p<0.001). **C)** Cd concentrations adjusted for liver fat% before the formation of yearly geometric mean values. The Coefficient of variation (CV) decreases from 63% to 59% and the lowest detectable trend (LDT) expected to be found with an 80% power during a study period of 10 years improves from 26% to 24%. The gain in power from adjustment is in this case considerably smaller compared to similar data from cod caught south east of Gotland, c.f. Fig.2.

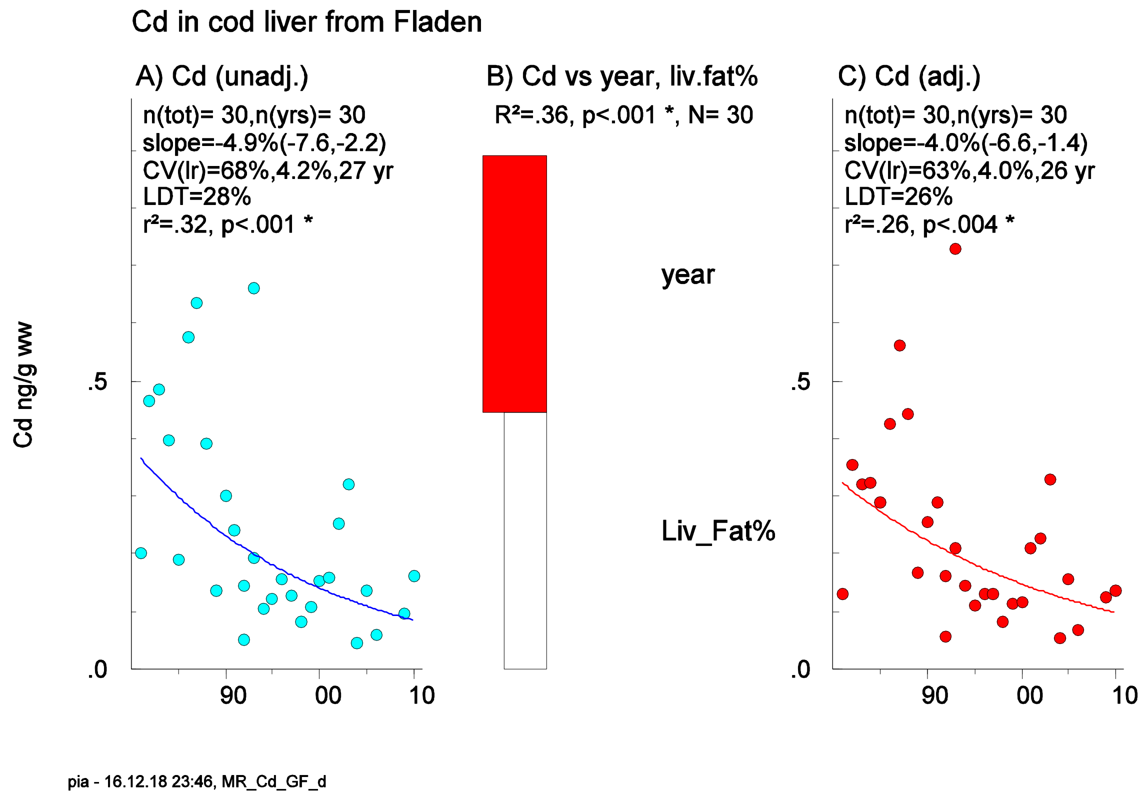

Figure S3. Temporal trend assessments based on pooled samples. **A)** Unadjusted pools. **B)** Multiple regression show that, unlike the cod from Gotland, logged cadmium concentrations expressed on a dry weight basis is not significantly correlated with the fat content in cod liver **C)** Pools adjusted for liver fat%. Despite the fact that the correlation is not significant, still the Coefficient of variation (CV) decreases somewhat from 68% to 63% and the lowest detectable trend (LDT) expected to be found with an 80% power during a study period of 10 years improves from 28% to 26%.

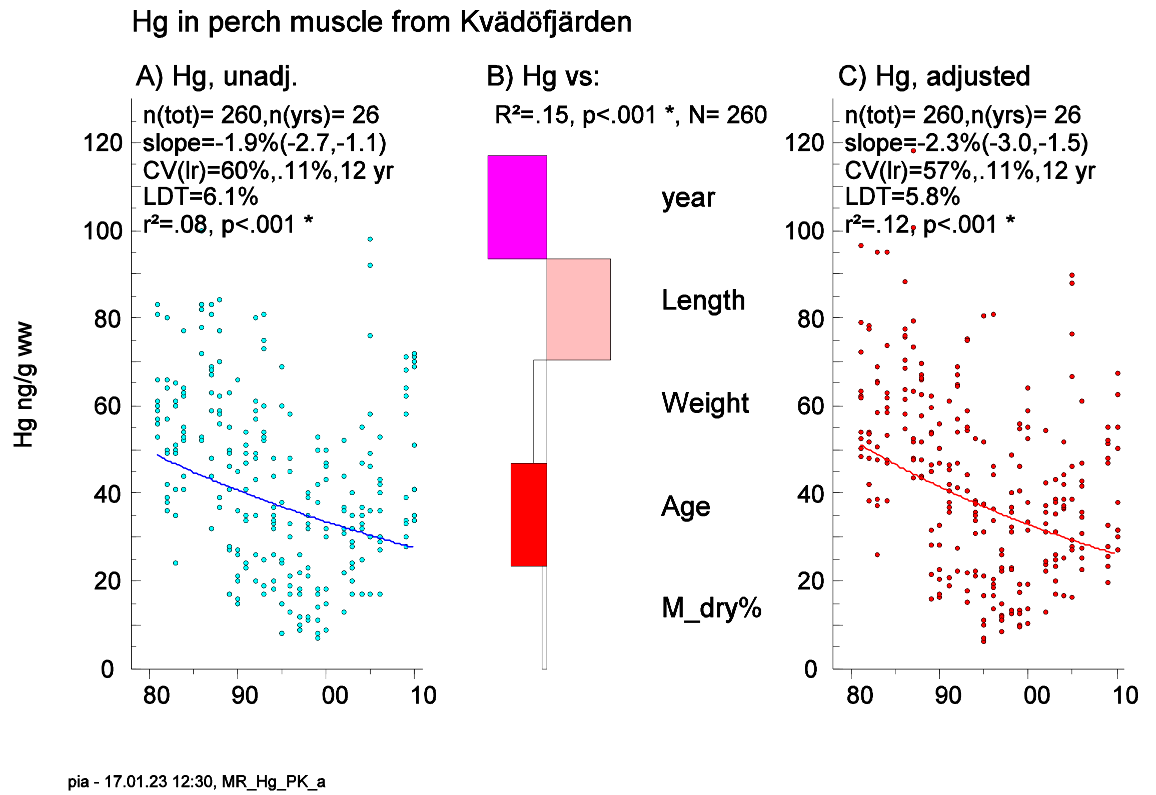

Figure S4. Temporal trend assessments of mercury concentration in perch muscle from Kvädöfjärden, based on individual specimens. **A)** Unadjusted Hg concentrations over time **B)** Multiple regression show that logged mercury concentrations expressed on a wet weight basis is significantly correlated with length ($p<0.05$) and age ($p<0.01$) The opposite directions of the correlations between Hg and length and age respectively, may seem confusing. Age is a course measure and more difficult determine with high precision than the length and to include both length and weight should maybe be avoided. **C)** Mercury concentrations measured in individual perch specimens adjusted for the variables listed in B. The Coefficient of variation (CV) decreases from 60% to 57% and the lowest detectable trend (LDT) expected to be found with an 80% power during a study period of 10 years improves from 6.1% to 5.8%. The log-linear trend fitted to the data is obviously not optimal. Visual inspection of the observed data indicates an initial decrease during the early 80-ies is followed by a log-linear increase starting at the turn of the century. This will lead to an increased between-year variation also after adjustment and possibly to *biased* correlations.

To apply adjustments for a temporal trend model that is wrong can be misleading.

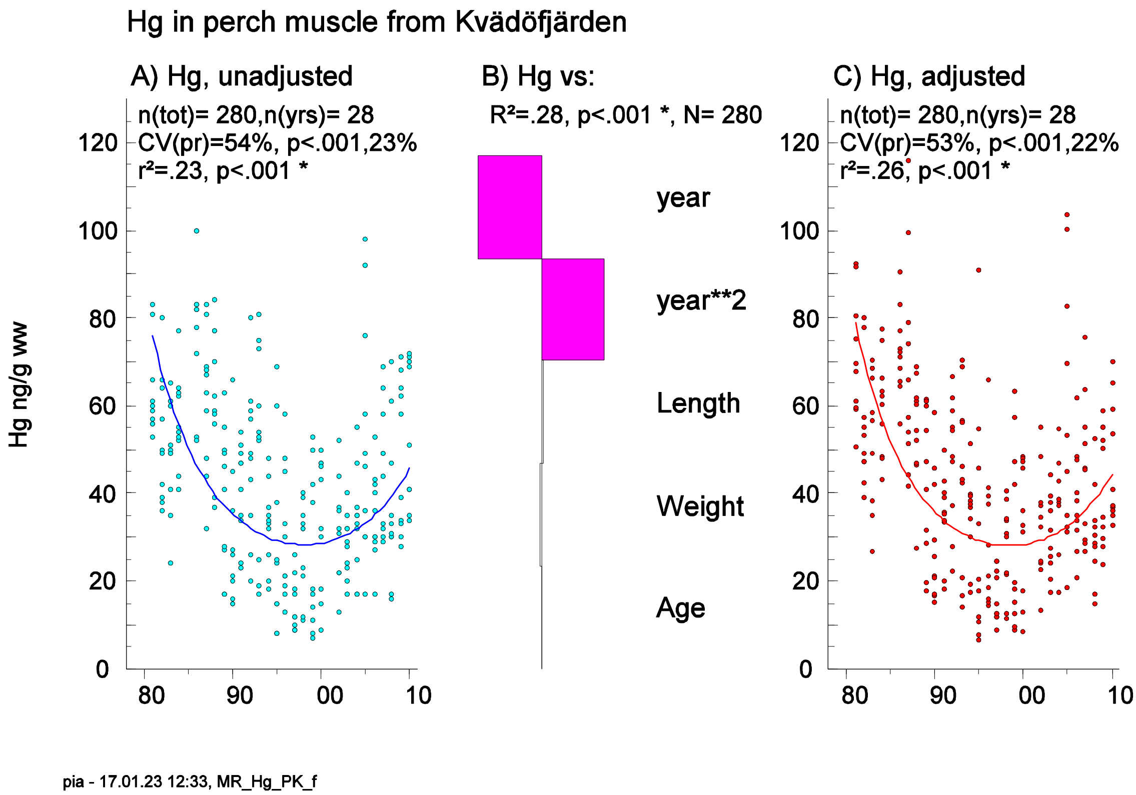

Figure S5. Temporal trend assessments of mercury concentration in perch muscle from Kvädöfjärden, based on individual specimens. **A)** Unadjusted Hg concentrations over time, quadratic polynomial regression applied (CV around the regression line decreases from 60% (see S4, above) to 54% for the quadratic model), **B)** Multiple regression show that logged mercury concentrations expressed on a wet weight basis is significantly correlated only with year ($p<0.001$) and year2 ($p<0.001$), **C)** Mercury concentrations measured in individual perch specimens adjusted for the variables listed in B. The Coefficient of variation decreases from 54% to 53%. Potential confounders like length (age) and weight do not contribute significantly to explain the unexplained part of the variation in mercury concentration. However, when adjusting the "pooled" samples, the effect is noticeable, see Fig. S8.

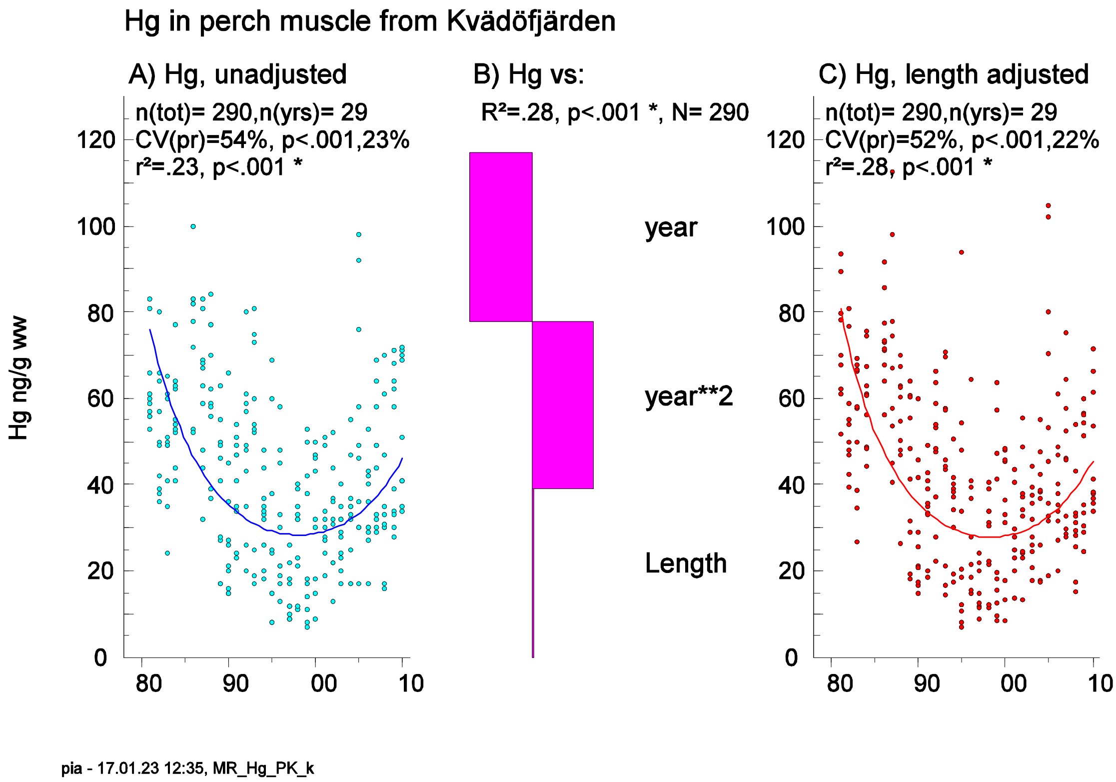

Figure S6. Temporal trend assessments of mercury concentration in perch muscle from Kvädöfjärden, based on individual specimens. **A)** Unadjusted Hg concentrations over time, quadratic polynomial regression applied (CV around the regression line decreases from 60% (see S4, above) to 54% for the quadratic model), **B)** Multiple regression show that logged mercury concentrations expressed on a wet weight basis is significantly correlated with year (p<0.001) and year2 (p<0.001) and length (less important but nevertheless significant in this reduced model (p<0.001), **C)** Mercury concentrations measured in individual perch specimens adjusted for the variables listed in B. The Coefficient of variation decreases from 54% to 52%

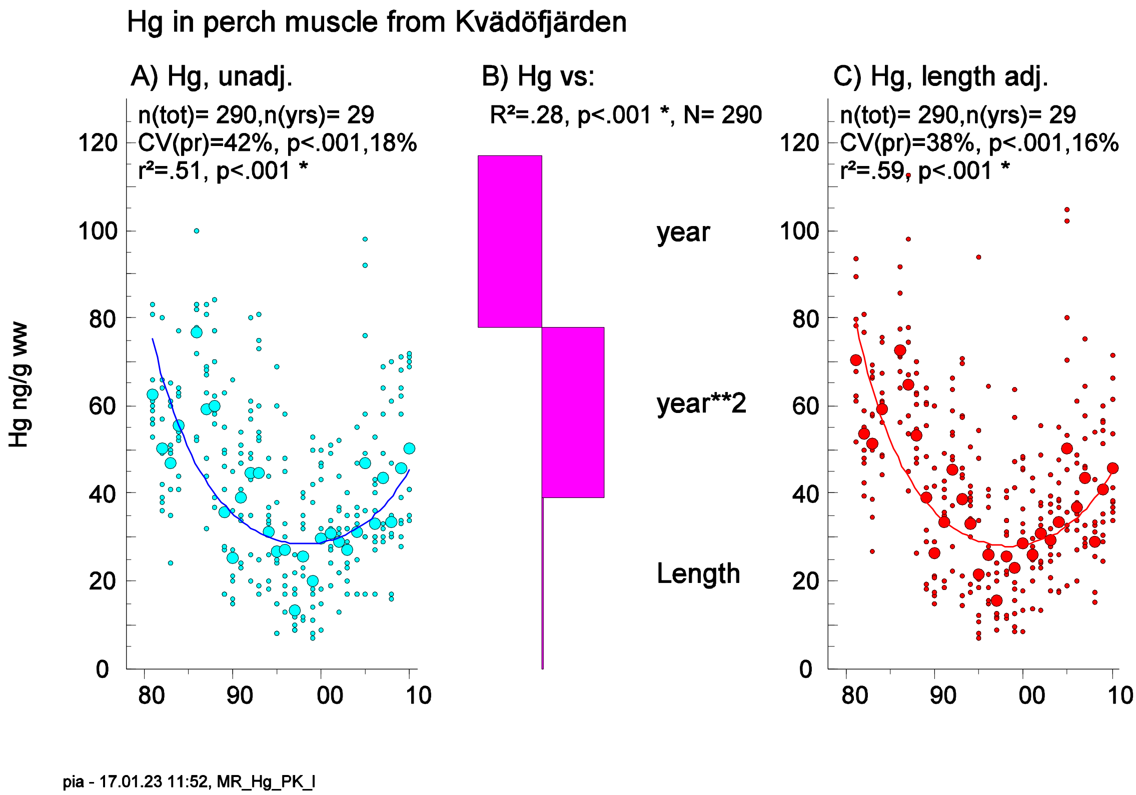

Figure S7. Temporal trend assessments based on yearly geometric mean values. **A)** Unadjusted yearly geometric mean values. **B)** Multiple regression show that logged mercury concentrations (geometric mean values) expressed on a wet weight basis show a change over time that can be described by a polynomial of the second degree and is also correlated with perch length ($p<0.001$). **C)** Hg concentrations adjusted for perch length before the formation of yearly geometric mean values. The Coefficient of variation (CV) decreases from 42% to 38%. The polynomial regression over time becomes stronger after the adjustment, r^2 increases from 51% to 59%.

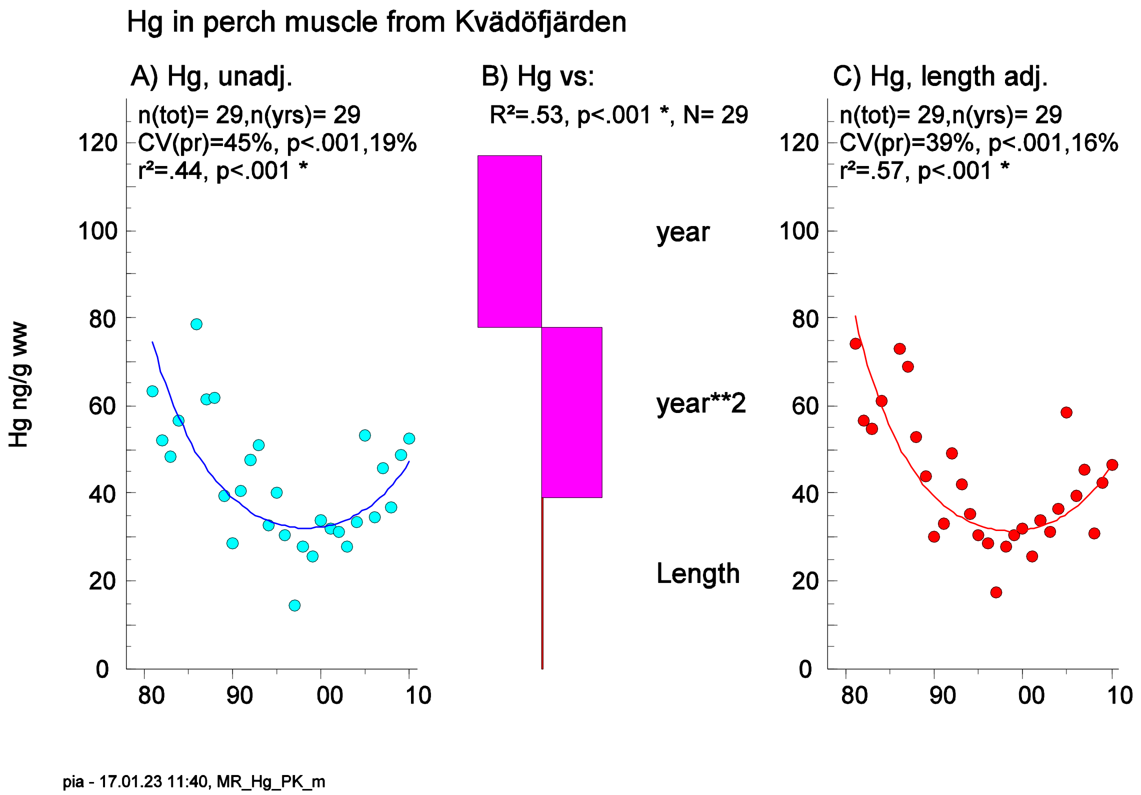

Figure S8. Temporal trend assessments based on pooled samples. **A)** Unadjusted pools. **B)** Multiple regression show that logged mercury concentrations (arithmetic mean values, simulating pooled samples) expressed on a wet weight basis show a change over time that can be described by a polynomial of the second degree and is also correlated with perch length ($p<0.01$) **C)** Pools adjusted for perch length. The Coefficient of variation (CV) decreases from 45% to 39%. The polynomial regression over time becomes stronger after the adjustment, r^2 increases from 44% to 57%.