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# Responses of Soil Nitrification Activities To Copper After A Moisture Stress

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**Research Article**

**Keywords:** successive stress, climate change, dry-rewetting, microbial indicator, toxicological thresholds, dose-response

**Posted Date:** November 22nd, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-988300/v1

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# Abstract

Some steps of the soil nitrogen cycle are sensitive to environmental pressures like soil moisture or contamination, which are expected to evolve during the next decades but such a double stress is not yet documented. This study aimed at assessing the importance of the soil moisture on the impact of copper (Cu) contaminations on the N cycling soil function using the potential nitrifying activities (PNA) as bioindicator. A loamy soil was first incubated 6 weeks in either 30, 60, or 90% of its water holding capacity (WHC) or alternating drought and rewetting periods. Thereafter, soil samples were exposed to a gradient of Cu concentrations through a bioassay. The dose-response curves of PNA in function of added Cu were modelled and we compared the different effective Cu concentrations (ECx) producing x % of PNA inhibition to highlight differences in threshold values. The preincubation moisture treatments significantly affected the PNA responses to the secondary Cu stress with, for instance, hormetic responses in all cases except for the dry-rewetting treatment. Small PNA inhibitions were estimated for high Cu doses in the soils with low water contents (30% WHC) or submitted to dry-rewetting cycles, contrarily to the patterns observed for the soils with high water contents (90% WHC) or submitted to a single period of drought. Overall, significant differences were found in estimated ECx values between moisture treatments.


# 1. Introduction

With the current scenarios of climate change, rainfall patterns are expected to change during the next decades (Lee et al., 2014) with more intense and longer drought periods followed by intense rainfalls. These modifications in the rainfall patterns may impact the soil water contents during critic periods. Excess of water or drought may affect soil moisture which is one of the main drivers of the soil microbial activity (Moyano et al., 2013). In parallel, human activities have dispersed significant quantities of contaminants into the environment, such as trace elements which are persistent and potentially toxic for the life soil biota (Giller et al., 2009). Nowadays, the contamination of soils by trace elements coming from atmospheric source or through agricultural practices has become a major concern at a global scale (Song et al., 2012). Trace element contaminations affect several environmental processes such as those performed by soil microorganisms (Giller et al., 2009) and soil microbiological indicators (abundance, diversity or activity) are hugely used to assess soil contamination. Microbial activities related to specific narrow niche processes are among the most sensitive endpoints to assess soil contaminant impacts (Broos et al., 2005). Therefore, bioassays measuring activities performed by microorganisms are useful to assess the severity of stress encountered by the soil ecosystem and possible outcomes on soil functions.

Microbial communities would encounter multi-stress of both global climate changes, soil contaminations and their combined effects, these last ones being less documented. Soils nitrifying communities have been shown to be highly sensitive to heavy metal stress as a Cu contamination (Ruyters et al., 2010; Smolders et al., 2001) as well as to soil moisture variations (Van Groenigen et al., 2015). However, effects of a first more or less mild stress on the response of microbial functions to a secondary stress are not well known, with reported increase or decrease of resistance depending on the stress (Philippot et al.,

2008; Rusk et al., 2004). In this context, it is difficult to predict the combined effect of climate change and contamination pressures on soil functions performed by soil organisms.

This work investigated the outcomes of an ecological stress ascribed to soil moisture on a chemical stress induced by copper (Cu). We focused on the effect of new-born exogenous Cu contaminations spiked to a soil which was first submitted to various moisture stresses: constant, small, or high soil moistures but also intermittent intense moisture stresses with drought and rewetting cycles. Cu was used as a model of contaminant because it is widely found in the soils in particular in Europe (Panagos et al., 2018) mainly due to its use as fungicide in agriculture. Cu concentration thresholds for soil management could vary largely depending on the agricultural policies. We also focused on PNA as an end-point. The nitrogen (N) cycle is an important contributor of emissions of greenhouse gases (GHG), particularly from agricultural sector. For instance, $N_2O$ is a powerful GHG with approximatively 256 fold the warning potential of $CO_2$ on a 100-year time horizon (Myhre et al., 2013). Moreover, N cycle is performed by a limited number of microorganisms, making it more sensitive to environmental pressures than C cycle (Broos et al., 2005). Also, N cycle is highly sensitive to soil moisture as nitrifying and denitrifying communities concur in soils depending of its oxygenation and thus of its moisture (Van Groenigen et al., 2015).

In this context, the aim of our study was to assess the effect of the soil moisture history - used here as one of the factors of climate change - on the impact of added Cu on potential nitrification - used here as a proxy of the soil nitrification process. Thus, a dose-response approach was carried out. First, absolute PNA (aPNA) responses after moisture and Cu stresses were quantitatively analysed. Then relative PNA (rPNA) responses compared to the no-added Cu sample were used to both 1) compare the pattern of the dose-response curves and 2) determine effective Cu concentrations inducing 5, 10, 20, and 50% (EC 5, 10, 20, 50) of decrease in rPNA. We hypothesized that 1) the pre-incubation periods under various moisture treatments affect the microbial community function related to the N cycle; 2) consequently the dose-response curves rPNA = f(added Cu) show different patterns with the various moisture pre-treatments; and 3) effective Cu concentrations are useful indicators to highlight differences in threshold values when a stress on stress occurs.

# 2. Materials And Methods

## 2.1 Soil sampling

The soil, described in Obriot et al., (2016) as a luvisol with 11g/kg of organic carbon and pH 6.9, was sampled in January 2017 in the control plot of the experimental Qualiagro site (48°87’N, 1° 97’E 17). This agricultural plot is not contaminated with Cu (no registered inputs since more than 20 years).Its Cu content is around 12 $mgCu.kg^{-1}$ consistent with the regional pedogeochemical background. Several fresh soil samples were pooled and immediately sieved at 5mm and stored at 4°C few days before building-up the microcosms to drive soil moisture regimes. Aliquots of this sieved soil were used to measure the fresh

soil moisture at the time of sampling which was 15% and the field capacity as maximum water holding capacity (WHC) which was set up at 32% (w/w).

2.2 Experimental setup

To evaluate the impact of soil moisture on the sensitivity of nitrification to Cu toxicity, a two steps experimental approach was carried out: a first pre-incubation period of five weeks was running under different moisture stresses before application of a secondary metal contamination stress during a 72h bioassay allowing evaluation of the absolute and relative potential nitrifying activities).

a) Microcosms to drive soil moisture regime

Five microcosms of about 500 g of soil were incubated in five plastic boxes maintained half-open during 35 days and submitted to different treatments. Starting from the known initial moisture, three microcosms were set up at 30%, 60% and 90% of the soil WHC. This WHC was then kept constant by weighting and were made in order to roughly span respectively limiting, optimal, and close to water saturation treatments for the microbial activity. Later on, these three treatments will be called “30%, 60% and 90%”, respectively. The two others microcosms were incubated with variable WHC in order to simulate two kinds of drought and dry-rewetting cycles. One was left for about 3 weeks dry period without water inputs (gentle air drying) until 10% of the WHC before progressively rewetting at 60% WHC, while the other was treated with alternative cycles of one-week dry period (10% of the WHC by air-drying) followed by one week near-saturation period (90% WHC). Drying was performed by natural evaporation and moisture control by weighting. These two samples will be called thereafter “Drought” (DO) and “Dry-rewetting” (DR). The concentrations of $N\text{-}NO_2$, $N\text{-}NO_3$, $N\text{-}NH_4$ and dissolved organic carbon (DOC) at the end of the pre-incubation period for the controls without added Cu and for each of the 5 moisture treatments are presented in table 1.

b) Bioassay to assess further Cu impact

After the pre-incubation period, the soil bioassays were immediately performed by measuring nitrate ($NO_3^-$) production rates over a short-term aerobic incubation in soil slurries (ratio soil:solution 1:6) with ammonium in excess and in the presence of gradients of Cu concentrations. The soil potential nitrification was then calculated on the basis of $NO_3^-$ measurement over the time period. The PNA bioassays were adapted from the methods proposed by (Tom-Petersen et al., 2004). Briefly, 5 g of fresh soil (approximately 4.7g of soil equivalent dry weight), were mixed in a Falcon® tubes with 29mL of a 10 mM HEPES buffer solution (hydroxyethyl piperazineethanesulfonic acid, Sigma-Aldrich, France) to maintain a constant pH under Cu spiking and nitrification activity, and containing the substrate $(NH_4)_2SO_4$ (3 mM) (Sigma-Aldrich, France). Then, 1mL of Cu solutions at different concentrations were added to reach final added Cu concentrations of 50, 100, 250, 500, 750, 1000 and 2000 $mgCu.\ kg^{-1}$ soil. Controls with only Milli-Q® water (Millipore) were also performed. Three independent samples (n = 3) from each pre-incubation treatment were ran in three concomitant bioassays for each Cu concentrations.

Following an initial horizontal shaking step (250 rpm, 10 min), the soil slurries were left incubated on a rotary shaker (150rpm), under aerobic treatments, at 25°C during 72h. After 10 min, 24 h and 72 h of incubation, 2 mL aliquots of the soil-solution mixture were transferred in Eppendorf® vials and centrifuged for 5 min at 13000 *g* at 4°C. The supernatants were collected and stored in microplates at –20 °C until analyses of $NO_3^-$ and $NO_2^-$ by colorimetric determinations, following the reduction of $NO_3^-$ in $NO_2^-$ by vanadium(III) and then the detection of $NO_2^-$ by the acidic Griess reaction (Miranda et al., 2001). Three different aliquots from each bioassay tube were analysed. Finally, the absolute value of PNA, aPNA in µg N-$NO_3$ $g^{-1}$ soil $h^{-1}$, was calculated on the basis of N-$NO_3^-$ + N-$NO_2^-$ concentrations measured at the different time steps. The points Cu=0 allowed us to verify that $NO_2^-$ contents were negligible (table 1), so that aPNA followed eq. 1, by checking the linear production rate between 2 h, 24 h and 72h:

$$(1)\ aPNA =$$

With

Vs: Volume of solution

W: Weight of fresh soil

T: Time of incubation

For each moisture treatment, the relative PNA values, rPNA values in %, were calculated by dividing aPNA for each added Cu level by the aPNA without added Cu.

2.3 Statistical analysis for the different moisture treatment on Cu comparison

Data of aPNA values between the different moisture treatments were compared for each Cu level with the use of Kruskal Wallis test followed by a post-hoc Dunnett test. Adjustment of p-value for multiple comparison was made by Holm procedure to limit false negatives.

Dose responses curves for rPNA were analysed with the DRC package, following recommendations of (Ritz 2010; Ritz et al., 2015). For the dose responses curves fitted by the same functions, the parameters were compared using the compParm function of the DRC package (see 2.4).

ECx comparisons were made through the estimated confidence intervals (IC), so that for each ECx the moisture treatment with overlapping IC was not significant different.

All analysis were conducted using R v4.1 (R Core Team 2021).

2.4 Dose-response curves analysis

To fit the dose-response curves, we have proceeded by different steps according to our aims of both estimate and compare the variations in rPNA due to Cu contamination stress under the different moisture stresses. Expected results are to provide EC5, EC10, EC20 and EC50 values as % response (at 95% confidence interval) expressed as rPNA for each moisture treatment.

For the first aim, i.e. estimating the variations in rPNA, the selection of the best dose-response model able to fit the experimental data was achieved by testing the following widely used models in ecotoxicology studies (Ritz et al., 2015): the log-normal (LN) dose-response model, or the log-logistic (LL) and their Weibull derivative (W1 or W2), or the hormetic models as the Cedergreen models (CD, tested with alpha =1 and alpha=0.25), the Gompertz (G) model, or the Braincousens models (BC.4 or BC.5 depending of the number of parameters to be fitted). Lower asymptotes were fixed to 0 and upper to 1 except for the hormetic models.

For the second aim, we considered that the comparison of the dose response curves and their parameters as a whole between all the moisture treatments better required using a common model for all the curves, contrarily to ECx determinations that required more precise fits to extract ECx values. We thus compared the five best models per moisture treatment according to AIC and logLik criteria and confirmed the choices by visual confidence interval accordance (limited confidence interval). From these five pre-treatments x 5 models we looked for a common model. If no common type of model was found powerful enough to extract ECx values, we selected more types of models for these extractions.

# 3. Results

3.1 Absolute PNA evolution under successive stresses

Fig 1 shows the mean, 1$^{st}$ and 3$^{rd}$ quartile of aPNA values obtained for all pre-incubation treatments (DR, DO and, 30, 60 and 90% WHC treatments) in function of the Cu concentration gradient.

The comparison between the control bioassay without Cu (Fig. 1, Cu 0) and the values in the presence of added Cu shows that the moisture pre-incubation treatments significantly affect the aPNA. Significant differences were found between aPNA for soils incubated under DR stress and soils incubated under 60% WHC, with aPNA values roughly smaller by one third in the soils submitted to dry and rewetting cycles (DR treatment).

The aPNA values decreased with increasing soil Cu concentrations and the moisture pre-treatment differently affected the aPNA inhibition by Cu stress. At the highest Cu doses (>1000 mgCu.kg$^{-1}$), no more differences in aPNA values could be observed whatever the moisture treatments. Therefore, above a threshold of 1000 mgCu.kg$^{-1}$, the effects on the nitrification processes were the same whatever the initial moisture pre-incubation.

3.2. Relative PNA evolution under successive stresses analysis with dose responses curves

3.2.a Selection of dose responses models for ECx estimation.

Suppl. table 1 gives the values of the criteria obtained for the five best fit of each of the dose-response curves: rPNA inhibition = f (total added soil Cu). Model selection based on the lowest value of AIC and higher value of LogLikelihood criteria showed that Cedergreen (alpha=1) and Braincousens or Weibull models are the best ones describing the inhibition of rPNA in function of total added Cu for incubation performed under constant moisture treatments (Supp Table 1a). Cedergreen and Braincousens models integrate hormesis parameters, so that modelling included our observations of an increase in rPNA at small Cu inputs in several moisture treatments (Fig. 2a)

If we consider the AIC criterion, inhibition of rPNA in DO incubated soils were better described by Braincousens or Weibull II and DR by Weibull I models (Suppl. table 1b). But for DO the Weibull fit indicators (AIC, Lack of Fit…) were close to those of Braincousens, and we chose using Weibull for homogeneity with the DR modelling to further extract ECx values. This means that rPNA inhibition of soils incubated under DO treatment  was poorly affected by the Cu gradient for low Cu concentrations. On the contrary, soils incubated under DR treatment are largely affected by variations of moderate Cu concentrations but poorly by the highest (Fig.1, 2b).

In order to compare the parameters of the dose-responses models selected taking into account their biological meaning, only one type of dose response model had to be selected. For that, we looked at using a single model whatever the pre-treatments. We also tested specifically the Cedergreen modelling function in the case of DO and DR conditions for two reasons: i) Braincousens and Cedergreen models are both hormetic models and ii) the Cedergreen fits were found acceptable compared to Braincousens for all the constant moisture treatments.

3.2.b Effect of pre incubation on dose-response curves.

The Cedergreen parameter models finally obtained are given in table 2, and are significantly different from 0 ($p < 0.05$) for all pre-incubation treatments except the hormesis effect in DR. This means that stimulation of the rPNA at low Cu concentration is not possible in the case of DR treatment (table 2, Fig 2b). No differences in the size of the hormetic effect (f parameter, table 2) were found between all the four other treatments. For these four other treatments, we were able to estimate the maximum estimated response (e.g. the maximum increase in the potential nitrifying activities) and associated Cu concentration. In all cases (30, 60, 90 %WHC and DO), the increase in rPNA at small doses of added Cu is limited to 5% (maximal response expected from 1.047 to 1.052 % of aPNA). The associated Cu concentration modelled by the Cedergreen fit was roughly twice higher for the 90% WHC and the DO treatment than for the 30 and 60% WHC treatments (22 and 26 $mgCu.kg^{-1}$ against 11and 14 respectively).

In parallel, the “b“ parameter which represents the steepness of the curve after hormesis is found significantly higher in the DO case than in the four other treatments, whereas it was found significantly smallest in the 30% WHC case. This means that for DO treatments the decrease in rPNA is higher for

smaller Cu concentration and that for 30 % WHC the decrease in rPNA is smallest with the increase in Cu concentration.

No straightforward interpretation of the “e” parameter could be draw as it only represents the low limit for the EC50 values and not a precise estimate. However, from the fitted results we may expet highest EC50 values with highest moisture incubation treatments and even more with DR and DO treatments (table 2).

3.3. Estimation of the effective Cu concentrations inducing PNA inhibitions

ECx extraction was made on the basis of Cedergreen models for the constant moisture treatment and on Weibull models for the treatment with various moisture (Weibull II for DO, Weibull I for DR). Table 3 gives the values of EC5, EC10, EC50 for each soil moisture status. For a given percentage of rPNA inhibition, we found significant differences in the estimated amounts of Cu inhibiting rPNA between the different moisture pre-treatments (Table 3). For instance, a 5% rPNA inhibition was predicted for a low soil Cu contents of 185 (±66) $mgCu.kg^{-1}$ for the 30% WHC pre-treatment but a higher soil Cu content of 405 (±88) $mgCu.kg\ soil^{-1}$ was predicted for soils pre-incubated under DO treatment. In parallel, a 20% rPNA inhibition was predicted for a low soil Cu content of 440 (±88) $mgCu.kg^{-1}$ for the 30% WHC pre-treatment but for a higher soil Cu content of 721 (±95) $mgCu.kg\ soil^{-1}$ for soils pre-incubated under DO treatment. These results suggest that drought is highly affecting for a subsequent Cu contamination compared to drought followed by a progressive rehumectation. This pattern is found again for all the x levels except for the 50% level. Taken into account the lower and upper values of EC5, we estimated that rather small Cu total concentrations added to soil around 185 $mgCu.kg^{-1}$ are able to induce a decrease in potential nitrifying activities when the soils is at 30% and 60% WHC but not for soils at 90% WHC or when soil is subjected to DO pre-treatment. In the same manner, we estimated that a value of around 400 $mgCu.kg^{-1}$ reduced rPNA by only 5% in the sample subjected to DO pre-treatment while about roughly 20% of rPNA inhibition was observed in the soil sample at 30% WHC.

Except for x = 50, the ECx values calculated for DO were always found higher than for DR. However, the differences were only significant in the case of EC5. On the other hand, and for a given pre-incubation, we observed no significant differences between the estimated EC5 and 10 (Table 3). For x = 50, the EC50 values were found significantly higher after DR pre-treatment (1763 ± 230 $mgCu.kg^{-1}$) than at 30% WHC (1220 ± 179 $mgCu.kg^{-1}$ for the 30% WHC, table 3), and tend to be higher for DR than for DO but with no significant differences.

# 4. Discussion

4.1. Use of PNA as indicator of successive moisture and chemical stress events

PNA is a frequently used indicator of soil contamination and of loss of soil functions (Broos et al., 2007; Hund-Rinke & Simon, 2008) despite its high variability in non-polluted soils (Sauvé et al., 1999). Here we submitted the soil samples first to a moisture stress before applying a secondary metallic stress, and we

focused on the potential soil nitrification activities response to these two conjugated stresses. Thus, aPNA measured in our bioassay is a reflection of the activity of the whole nitrifying community selected through microcosm moisture treatments and its ability to resist to a subsequent Cu stress. Dry-rewetting cycles for soils have been reported to enhance C and N mineralization due to nutrient flush with rewetting (Birch, 1958) but with various effects on $NO_3^-$ concentrations depending on the soil use or on the number of dry-rewetting cycles (Fierer & Schimel, 2002). Our hypothesis was that the various moisture stresses do not select the same communities inducing different nitrate production abilities. This is consistent with our results showing that rPNA values were affected by the pre-incubation moisture with roughly 30% inhibition in the soils incubated under DO or DR treatments compared to the soils incubated at a constant moisture.

PNA measurements can also be used as a sensitive tool to define ecotoxicological guidelines (Broos et al., 2005). In the literature, it is often noticed that EC50 values are difficult to obtain mostly due to the fact that dose-response curves are rarely fully complete until the end-point being zero. The determination of EC50 values by extrapolation could thus be less interesting and more ambiguous than the determination of threshold with smallest ECx with x<50. In our experiments, we only measured 2 cases where rPNA was inhibited higher than 50% whatever the pre-incubation treatment, and we never reached a plateau in rPNA inhibition. Despite the fact that satisfying at least one of these two conditions is a guarantee to provide accurate estimates (Sebaugh, 2011), our results showed limited incertitude around the estimated ECx values. For the high EC values we obtained an incertitude of 14% in added Cu in mean compared to the 31% of total added Cu in mean concentration for the small EC (EC5).

The beginning of the dose-responses fits for the small concentrations of added Cu provided hormesis effects with different ranges (table 2 and estimated max PNA). This variability in the range of the small dose effects is not captured by ECx values with small x, but rather lead to large incertitude's in EC5 determinations. Such incertitude decreased for EC10 and EC20. Also, if ECx data are useful threshold providing values easily transferable in terms of contamination management, their determination after modelling may be not sufficient to identify smallest dose effects. Finally, the use of PNA as indicator of successive moisture and chemical stress events was powerful in our conditions to highlight an effect of moisture on the response of a soil to a Cu stress, and to allow comparison of ECx for different moisture pre-treatment in particular for x = 20.

4.2. Effects of a double stress as preliminary moisture stress followed by a Cu stress

In the present laboratory study the Cu stress was applied secondary of the dry-rewet or constant moisture events. In the field, it can also happen that dry rewet events occur in Cu contaminated soils, which is another scenario as the one we studied.

In this framework, our results show that moisture soil history changes the soil PNA response to a supplementary stress. Soil samples submitted to a single long dry cycle (DO) or staying at 90% WHC seemed to be more tolerant to a subsequent Cu stress than for those submitted to dry-rewetting cycles

periods (DR) or 30% WHC (Fig. 1, table 3). The 60% WHC microcosms were found in-between these two cases. This can be clearly seen through the higher EC5 to EC20 values for DO soils.

The rather surprising result concerning DO could be due to our experimental design where the last week the sample was gently moistened back from 10 to 60%WHC. For DR, we hypothesized that this incubation strongly selected communities. Indeed, it has been shown that less diverse microbial communities may be more sensitive to subsequent stress (Hallin et al., 2012). The hypothesis of a highest primary stress in the soils incubated under DR treatment is somehow supported by the absence of hormesis, whereas in the four others treatments low Cu concentrations induced slight increases in PNA, suggesting that microbial communities could have enough resources to do so.

For the soils incubated at 30% WHC, no effect of pre-incubation period was noticed on PNA without added Cu but we noticed a high sensitivity to Cu contamination that could be due either to a low pool of microorganisms resistant to Cu or to a high level of Cu bioavailability.

On the contrary, the soils incubated at 90% WHC showed high resistance to Cu that could either be due to a lower Cu availability in the soil solution or to a higher pool of soil bacterial communities resistant to Cu. To disentangle between these hypotheses, it could be interesting to design the experiments by including proxy of Cu availability as well as characterization of the communities (structure or diversity) after the first stress to assess potential differences in the modifications of microbial communities between the pre-incubations.

Finally, we observed that the aPNA values were found different between the constant and various moisture pre-treatments only for the lowest part of the dose-response curves, thus for low added Cu concentrations, and became similar at highest added Cu concentrations (Fig. 1). Such a result suggests that the pre-incubation patterns have modified the sensitivity (Cu concentration initializing a loss of function) but not the resistance (disappearance of the function) of aPNA. However, we cannot conclude if the aPNA measured under 2000 $mgCu.kg^{-1}$ are minimal values (Fig. 1) due to microbial highly resistant groups or if higher levels of Cu would have reduced aPNA.

# 5. Conclusion

Our study showed that pre-incubated soils at low moisture (30% WHC) were more sensitive to a secondary Cu stress than those pre-incubated at a higher moisture (60 and 90% WHC), with EC5 values defined respectively around 185 (±66), 231 (±75) and 349 (± 96) $mgCu.kg^{-1}$. Soils submitted to gentle drought (DO) then gentle rewetting were surprisingly less sensitive to Cu with a EC5 value at 404 (± 88) $mgCu.kg^{-1}$ whereas soils submitted to drastic changes in moistures (DR) lost nitrification activities as soon as low amounts of Cu (EC5 = 189 (±78) $mgCu.kg^{-1}$) indicating their sensitivity to the second stress. These Cu amounts in agricultural parcels are not seldom with Cu inputs through fertilizers or pesticides resulting from several years of cumulative inputs (Panagos et al., 2018). Nevertheless, our results showed that climate and particularly rainfall patterns have to been considered because micro-organisms and the

soils functions they provide may be differentially affected by Cu stress depending on the soil moisture history. Our results showed that differences in PNA values between the moisture histories we studied decrease when the Cu contamination increases. We also show that ecotoxicological studies based on ECx determination should be complete by dose-responses curves fitting analysis that highlight more precise patterns. Indeed, this permit us to emphasize small increases in PNA for low added Cu concentration close to 20 $mgCu.kg^{-1}$ for four to five preincubation treatments that were not exemplified through ECx determinations. Considering the key roles of the soil N emissions in the GHG emissions, our results could be useful to provide a combined estimation of nitrification and denitrification fluxes and activities of the microbial communities involved in these functions in the context of climate change and soil contamination.

# Declarations

Competing Interests: authors have no competing interests to declare.

Aknowledgment : the author thank the LabeX BASC (ANR-11-LABX-0034) for its funding through the CONNEXION project. Laura Sereni also thanks the ENS for her grant. The author thanks Amélie Trouvé for her help in soil data analysis, Sébastien Breuil for soil processing and Christelle Marrauld for her help in the bioassay design.

Ethical Approval: this paper has no ethical issue (not working on animal or human issues)

Consent to Participate: all authors consent to participate to this scientific publication done with the results of the study done within the framework of the CONNEXION project funded by the Agence National de la Recherche

Consent to Publish: all authors consent to publish in Environmental Science and Pollution Research the results of experimentations done within the framework of the CONNEXION project funded by the Agence National de la Recherche

-Authors Contributions: the authors contributed as follows:

Laura Sereni: Methodology, Formal analysis, Data processing, Writing original draft.

Bertrand Guenet: Methodology, conceptualization, writing review and editing, supervision

Olivier Crouzet: Methodology, conceptualization, writing review and editing, supervision

Charlotte Blasi: Experimentations and draft initialization

Isabelle Lamy: Methodology Conceptualization, Writing review and editing, Supervision Project administration, Funding acquisition.

Funding: the study was found by the Agence Nationale de la Recherche with the LabEx BASC (ANR-11-LABX-0034) and the CONNEXION project.

Competing Interests: authors have no competing interests to declare.

Availability of data and materials: Data and code are available upon request to the authors

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# Tables

Table 1: Mean and standard error (Std error) of concentration of N species and dissolved organic carbon (DOC) for the 3 replicants of each pre incubation moisture condition at the end of the preincubation showing negligible values of $N\text{-}NO_2$.

| | $N\text{-}NO_3$ (µg / g de sol) | | $N\text{-}NO_2$ (µg / g de sol) | | $N\text{-}NH_4$ (µg / g de sol) | | |
|---|---|---|---|---|---|---|---|
| Moisture condition | mean | Std error | mean | Std error | mean | Std error | DOC [mg.L-1] |
| 30 | 14.64 | 0.53 | 0.14 | 0.02 | 4.26 | 0.23 | 5.45 |
| 60 | 18.77 | 0.05 | 0.16 | 0.03 | 6.84 | 0.10 | 4.61 |
| 90 | 23.92 | 0.32 | 0.21 | 0.00 | 9.91 | 2.39 | 4.01 |
| DO | 27.43 | 2.06 | 0.29 | 0.08 | 6.23 | 1.07 | 4.41 |
| DR | 29.34 | 0.86 | 0.22 | 0.08 | 4.03 | 0.82 | 3.73 |

Table 2. Estimated coefficients and standard error for the Cedergreen model used to fit all the dose-response curves of rPNA against total added Cu concentration in the form

$$rPNA = \frac{1+f*\exp\left({}^{-1}/_{Cu\ tot}\right)}{1+\exp\left(b*\log(Cu\ tot)-\log(e)\right)}.$$

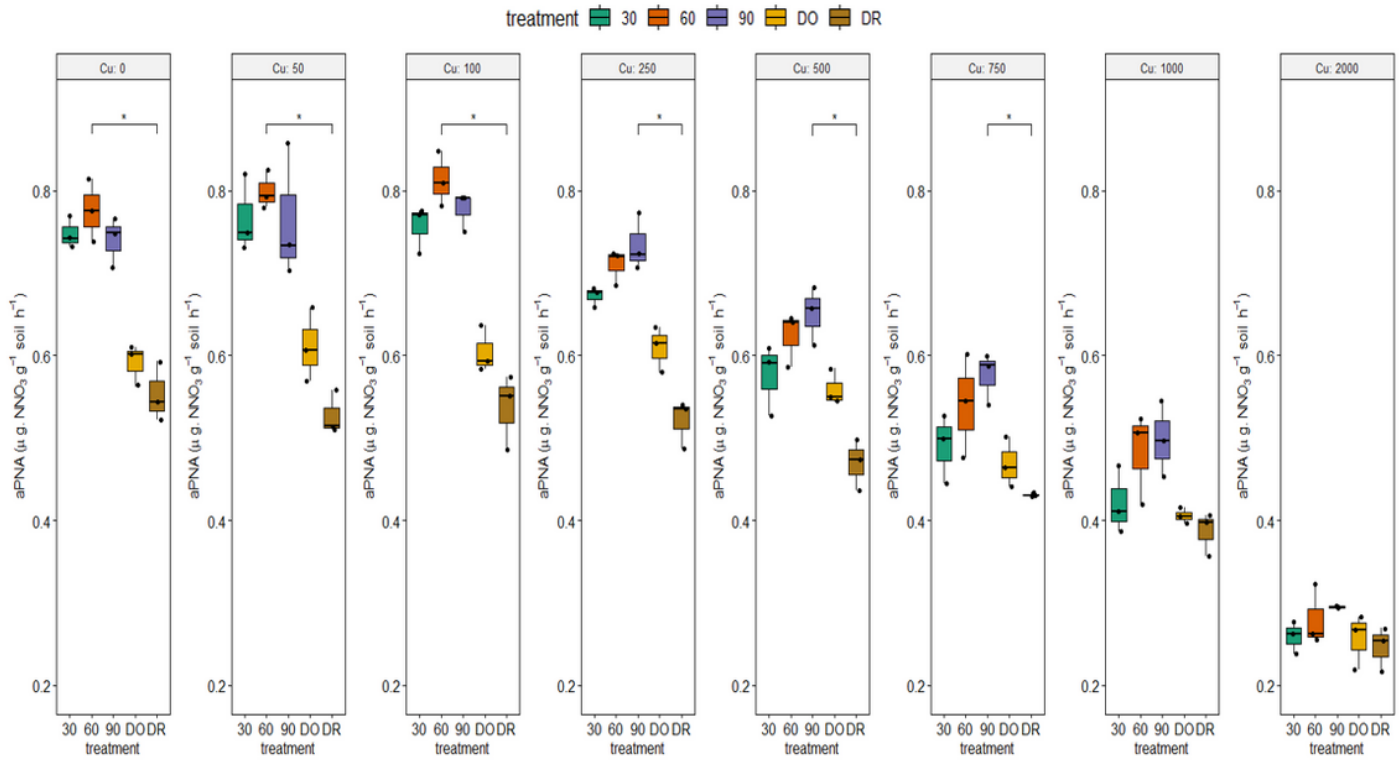
p-value are reported for test against 0. Last column refers to comparison of the 5 pre treatment for each coefficient. Coefficient sharing the same letter in the last column are not statistically different.

| coefficient | Estimate | Standard Error | p-value | Moisture Condition | Statistical comparisons |
|---|---|---|---|---|---|
| b | 1.22 | 0.13 | 1E-15 | 30 | a |
| | 1.28 | 0.13 | 1E-15 | 60 | ab |
| | 1.56 | 0.15 | 8E-18 | 90 | b |
| | 1.67 | 0.15 | 5E-19 | DO | bc |
| | 1.59 | 0.19 | 1E-13 | DR | b |
| e | 1120.2 | 75.6 | 9E-28 | 30 | a |
| | 1243.8 | 78.0 | 3E-30 | 60 | a |
| | 1450.4 | 77.3 | 1E-35 | 90 | cd |
| | 1558.4 | 81.6 | 3E-36 | DO | de |
| | 1805.5 | 114.4 | 9E-30 | DR | e |
| f | 0.056 | 0.029 | 0.06 | 30 | a |
| | 0.060 | 0.027 | 0.02 | 60 | a |
| | 0.053 | 0.021 | 0.01 | 90 | a |
| | 0.051 | 0.020 | 0.008 | DO | a |
| | -0.030 | 0.020 | 0.1 | DR | NS |

Table 3: Effective Cu concentration ECx inducing x% of rPNA inhibition with x = 5, 10, 20, or 50%. ECx estimates are in $mgCukg^{-1}$ soil and derivate from the functions cedergreen (method= CD) or weibull (method= W) taken into account the total added Cu concentrations. Lower and upper estimated values (95% CI) are given in $mgCukg^{-1}$ soil; moistures of 30, 60 and 90 refer to conditions of pre-treatments with 30, 60 and 90 % WHC, DR to dry rewet and DO to dry only. Last column refers to comparison of the 5 pre treatment for each x level. Coefficient sharing the same letter in the last column are not statistically different.

| X level | Moisture | Estimate | Lower | Upper | Method | Comparison |
|---|---|---|---|---|---|---|
| 5 | 30 | 184.9 | 119.2 | 250.7 | CD | a |
| | 60 | 231.3 | 156.6 | 306.1 | CD | a |
| | 90 | 348.8 | 253.0 | 444.7 | CD | ab |
| | DO | 404.8 | 316.8 | 492.7 | W2 | b |
| | DR | 189.4 | 111.6 | 267.1 | W1 | a |
| 10 | 30 | 265.8 | 193.8 | 337.8 | CD | a |
| | 60 | 323.4 | 241.3 | 405.4 | CD | ab |
| | 90 | 465.4 | 366.2 | 564.7 | CD | b |
| | DO | 516.9 | 425.2 | 608.6 | W2 | b |
| | DR | 350.9 | 249.7 | 452.1 | W1 | ab |
| 20 | 30 | 439.8 | 352.0 | 527.6 | CD | a |
| | 60 | 517.8 | 421.4 | 614.2 | CD | ab |
| | 90 | 693.1 | 586.8 | 799.3 | CD | b |
| | DO | 721.1 | 626.3 | 815.9 | W2 | b |
| | DR | 667.6 | 552.8 | 782.4 | W1 | b |
| 50 | 30 | 1221.9 | 1042.8 | 1401.0 | CD | a |
| | 60 | 1358.9 | 1155.7 | 1562.0 | CD | ab |
| | 90 | 1548.7 | 1329.0 | 1768.5 | CD | ab |
| | DO | 1577.7 | 1374.3 | 1781.1 | W2 | b |
| | DR | 1763.4 | 1533.1 | 1993.7 | W1 | b |

# Figures




## Figure 1

Mean, 1st and 3rd percentile of aPNA measured at the end of the bioassay for each incubation treatment and added Cu concentration. Preincubation treatment are represented per color with 30%WHC in green, 60% in orange, 90% in purple, DR in yellow and DO in brown. For each Cu level, post hoc Dunett test with Holm procedure was applied. Significant differences in PNA between moistures treatments are indicated by brackets branches and notated with stars (* : p.v <0.05)

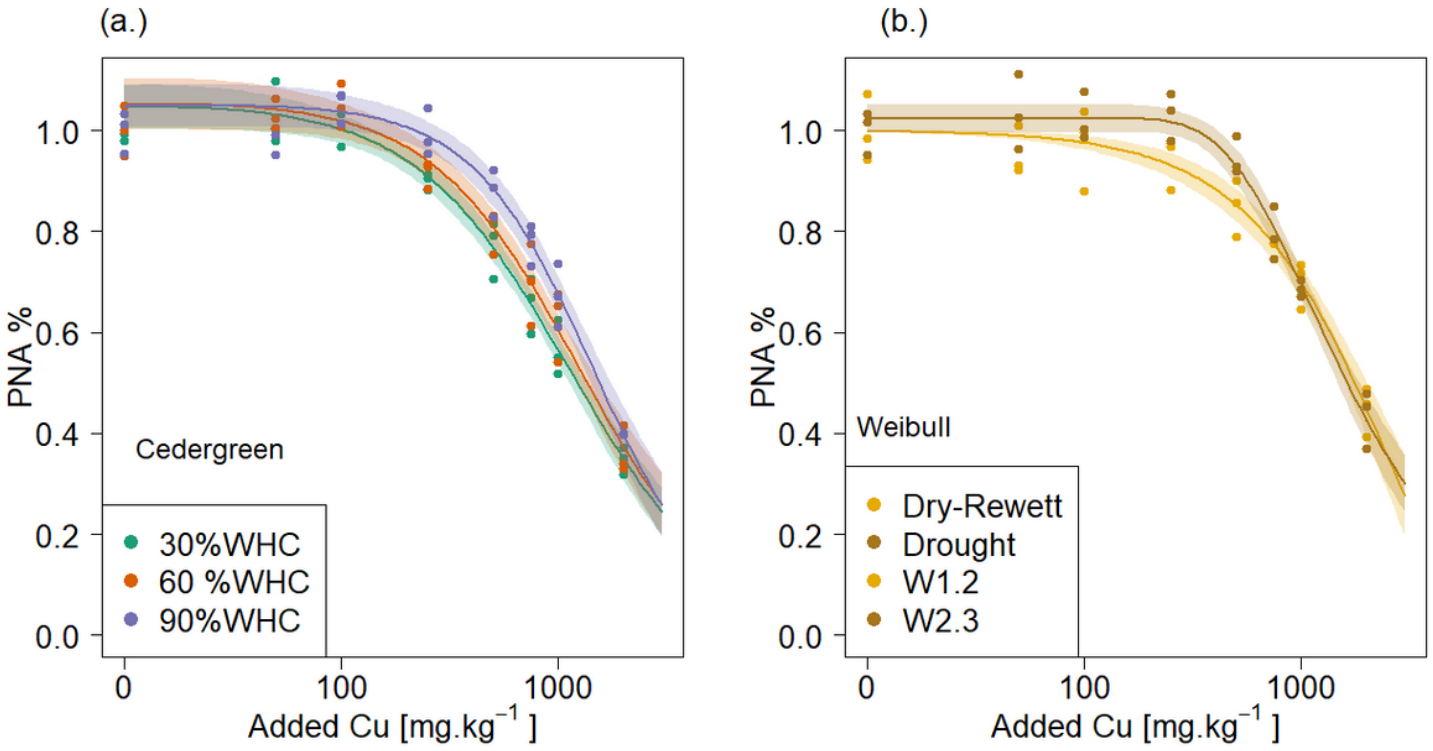



**Figure 2**

Fit of the selected function on the AIC criteria to model rPNA responses to added Cu with 95% confidence interval. (a). In the case of the incubation performed at constant moisture (30% in green, 60% in orange and 90% WHC in purple) with fit of Cedergreen functions. (b). In the case of the incubation performed under DR (dry rewet, yellow) and DO (dry only, brown) moisture treatment with fit of Weibull functions. Weibull model of the first type was used in the case of the dry-rewet incubation and Weibull model of the second type was used in the case of the dry only treatment.

# Supplementary Files

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