Impact of bio-palladium nanoparticles (bio-Pd NPs) on the activity and structure of a marine microbial community

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Corresponding Author: Dr. Giulio Zanaroli, Ph.D.

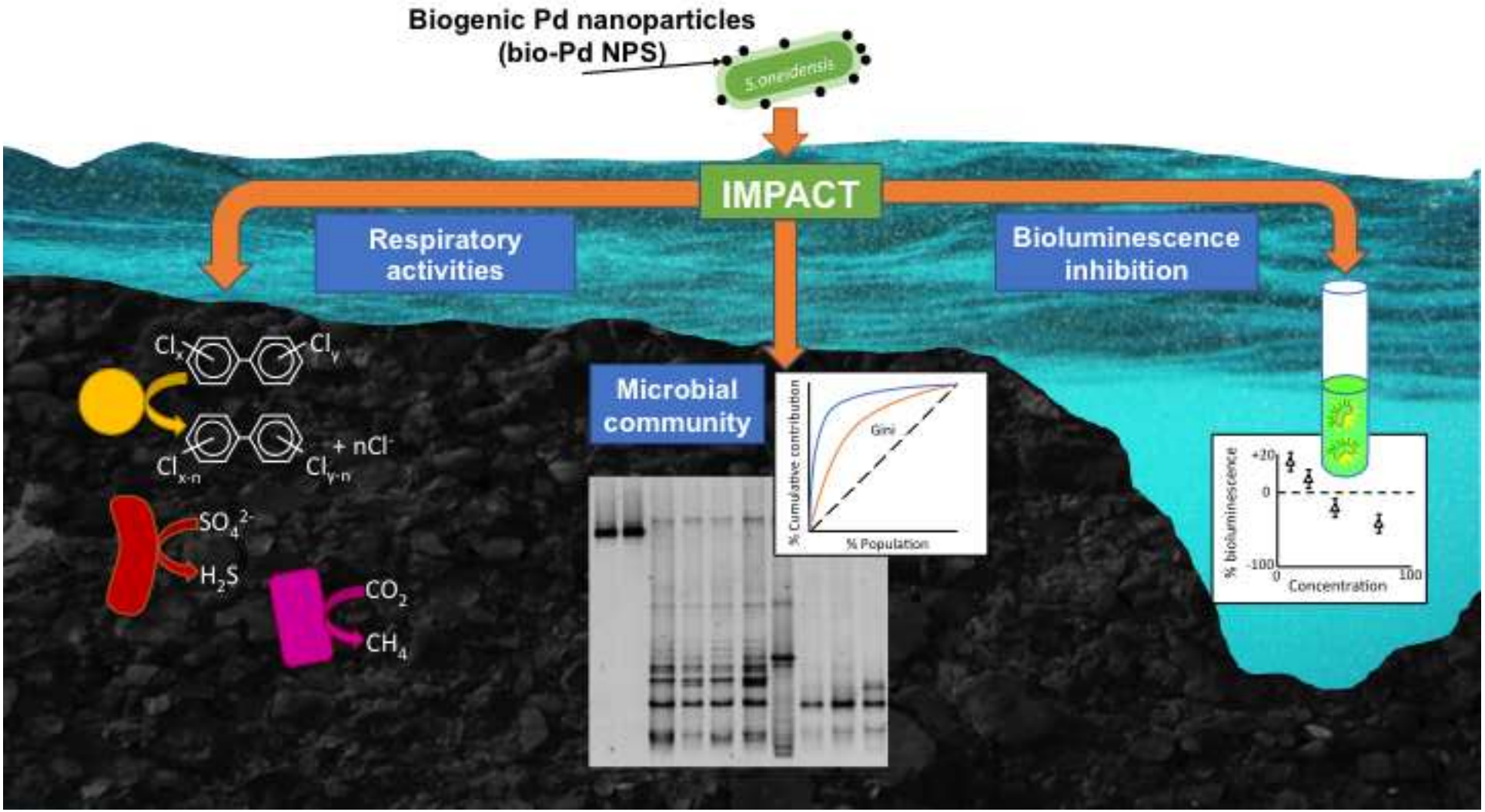
Corresponding Author's Institution: University of Bologna

First Author: Andrea Nuzzo

Order of Authors: Andrea Nuzzo; Baharak Hosseinkhani; Nico Boon; Giulio Zanaroli, Ph.D.; Fabio Fava

Abstract: Biogenic palladium nanoparticles (bio-Pd NPs) represent a promising catalyst for organohalide remediation in water and sediments. However, the available information regarding their possible impact in case of release into the environment, particularly on the environmental microbiota, is limited. In this study the toxicity of bio-Pd NPs on the model marine bacterium V. fischeri was assessed. The impacts of different concentrations of bio-Pd NPs on the respiratory metabolisms (i.e. organohalide respiration, sulfate reduction and methanogenesis) and the structure of a PCB-dechlorinating microbial community enriched form a marine sediment were also investigated in microcosms mimicking the actual sampling site conditions. Bio-Pd NPs had no toxic effect on V. fischeri. In addition, they had no significant effects on PCB-dehalogenating activity, while showing a partial, dose-dependent inhibitory effect on sulfate reduction as well as on methanogenesis. No toxic effects by bio-Pd NPs could be also observed on the total bacterial community structure, as its biodiversity showed a NPs dose-dependent increase compared to the not exposed community. In addition, resilience of the microbial community to bio-Pd NPs exposure was observed, being the final community organization (Gini coefficient) of samples exposed to bio-Pd NPs similar to that of the not exposed one. Considering all the factors evaluated, bio-Pd NPs could be deemed as non-toxic to the marine microbiota in the conditions tested. This is the first study in which the impact of bio-Pd NPs is extensively evaluated over a microbial community in relevant environmental conditions, providing important information for the assessment of their environmental safety.

*Graphical Abstract



Highlights:

- Bio-Pd NPs are deemed not toxic in standard *V. fischeri* ecotoxicity test
- Impact of Bio-Pd NPs tested on microbial community in its native sediment and water
- Bio-Pd NPs impact is limited to few respiratory activities
- Microbial community is resilient to bio-Pd NPs

Impact of bio-palladium nanoparticles (bio-Pd NPs) on the activity and structure of a marine microbial community

Andrea Nuzzo[a], Baharak Hosseinkhani[bł], Nico Boon[b], Giulio Zanaroli[a*], Fabio Fava[a]

a) Department of Civil, Chemical, Environmental and Materials Engineering, University of Bologna, Via Terracini 28, 40131 Bologna, Italy

b) Center for Microbial Ecology and Technology (CMET), Ghent University, Coupure Links 653, B-9000 Gent, Belgium

ł present address: Hasselt University, Biomedical research institute, Martelarenlaan 42, 3500 Hasselt, Belgium

* Corresponding author. E-mail: giulio.zanaroli@unibo.it ; Telephone: +39-051-2090317; Fax: +39-051-2090322

Abstract

Biogenic palladium nanoparticles (bio-Pd NPs) represent a promising catalyst for organohalide remediation in water and sediments. However, the available information regarding their possible impact in case of release into the environment, particularly on the environmental microbiota, is limited. In this study the toxicity of bio-Pd NPs on the model marine bacterium *V. fischeri* was assessed. The impacts of different concentrations of bio-Pd NPs on the respiratory metabolisms (i.e. organohalide respiration, sulfate reduction and methanogenesis) and the structure of a PCB-dechlorinating microbial community enriched form a marine sediment were also investigated in microcosms mimicking the actual sampling site conditions. Bio-Pd NPs had no toxic effect on *V. fischeri*. In addition, they had no significant effects on PCB-dehalogenating activity, while showing a partial, dose-dependent inhibitory effect on sulfate reduction as well as on methanogenesis. No toxic effects by bio-Pd NPs could be also observed on the total bacterial community structure, as its biodiversity showed a NPs dose-dependent increase compared to the not exposed community. In addition, resilience of the microbial community to bio-Pd NPs exposure was observed, being the final community organization (Gini coefficient) of samples exposed to bio-Pd NPs similar to that of the not exposed one. Considering all the factors evaluated, bio-Pd NPs could be deemed as non-toxic to the marine microbiota in the conditions tested. This is the first study in which the impact of bio-Pd NPs is extensively evaluated over a microbial community in relevant environmental conditions, providing important information for the assessment of their environmental safety.

Keywords

Bio-Pd NPs, Nanoparticles, Ecotoxicity, Marine sediment, Bacterial community structure

Capsule

Bio-Pd NPs are non-toxic towards *V.fischeri* and have a limited impact on a marine microbial community in site-mimicking microcosms.

Introduction

Nanoremediation is the application of reactive nanoparticles (NPs) for the detoxification of environmental matrices from pollutants (Karn et al., 2009); a long list of different nanomaterials have been applied so far in nanoremediation of contaminated water and soils, such as nanoscale zero-valent iron (nZVI) (Theron et al., 2008; Zhang, 2003), Pd/Fe bimetallic NPs, carbon nanostructures and many more (Theron et al., 2008). These NPs show very high reactivity and different properties compared to their bulk counterparts, due to their size and innovative surface coatings, allowing efficient and controllable degradation activities, especially against recalcitrant pollutants (Macé et al., 2006; Serrano, 2010). NPs of zerovalent iron represent a typical example of this approach, especially for the decontamination of groundwater from organic solvents, pesticides, dyes and polychlorinated hydrocarbons as well as inorganic ions (Zhang, 2003); more recently, other metal NPs have emerged as novel catalyst in nanoremediation approaches, called zerovalent palladium nanoparticles (Pd-NPs).

Pd nano-catalysts were efficiently used for reductive dechlorination of several contaminants (Ukisu and Miyadera, 2003). Pd-NPs catalyse the reductive dehalogenation of the contaminant through the development of radical cathodic hydrogen which chemically dehalogenates the molecule (Choi et al., 2009; De Windt et al., 2005); additionally, NPs of zerovalent Pd can be obtained through bioprecipitation in bacterial cultures and they are referred to as *biopalladium nanoparticles* (bio-Pd NPs) (Baxter-Plant et al., 2003; De Windt et al., 2005). Bio-Pd NPs have been effectively used for the degradation/transformation of contaminants such as heavy metals, halogenated organic solvents and pesticides in different environments, such as groundwater, wastewater, air, soil, sediments (De Corte et al., 2012) constituting promising catalysts (Hennebel et al., 2009) in an effective cradle to cradle approach which combine recovery of this precious metal from wastewater and a sustainable approach in pollutant nanoremediation (Hennebel et al., 2012).

Among halogenated compounds, polychlorinated biphenyls (PCBs) are persistent organic pollutants with toxic effects on all trophic levels, including humans (Safe, 1993). Due to their high hydrophobicity, recalcitrance to biodegradation, biomagnification capabilities throughout the food chain and toxic effects on both the environment and the human health, PCBs are still included in the Priority Organic Pollutants list (Stockholm Convention, 2004). After more than 30 years from worldwide production banishment, PCB pollution is still present in marine environments, especially in sediments (Fernández and Grimalt, 2003). In these environmental compartments some microorganisms of the phylum *Chloroflexi* are able to use them as final electron acceptor. Through this activity,

chlorine atoms are sequentially removed from the highly chlorinated PCB congeners leading to the accumulation of lower chlorinated ones which have usually lower toxicity and can be more easily degraded in aerobic conditions (Sowers and May, 2013; Zanaroli et al., 2015). This process known as microbial reductive dechlorination, has been mainly studied in freshwater sediment cultures (Bedard, 2008; Field and Sierra-Alvarez, 2008; Wiegel and Wu, 2000), whereas less is known regarding these activities in marine sediments (Fava et al., 2003a, 2003b; Zanaroli et al., 2015, 2006). In addition, microbial reductive dechlorination processes are extremely slow (Wiegel and Wu, 2000) and enhancement strategies must be developed for bioremediation approaches to compete with the traditional remediation technologies, which are environmentally and economically unsustainable (Khan et al., 2004; Perelo, 2010).

Bio-Pd NPs have been recently suggested as a promising approach to enhance PCB degradation also in marine environments, where they can be synthesized by some indigenous marine microorganisms (Hosseinkhani et al., 2014b). Using these biologically precipitated NPs, a complete dechlorination of TCE was achieved in synthetic marine water and marine slurries of water and sediments (Hosseinkhani et al., 2014a, 2014b) as well as an extensive dechlorination of Aroclor 1254 PCBs to mainly monochlorobiphenyls (Hosseinkhani et al., 2015). However, concerns are rising around the environmental remediation approaches using these NPs (Grieger et al., 2010; Karn et al., 2009; Otto et al., 2008; Sánchez et al., 2011); metal NPs might have toxic effects on humans and on the environment, due to their small dimensions, which allow them to penetrate easier and faster into the cell space (Jiang et al., 2009; Schrand et al., 2010). Pd- NPs, in particular, have an inhibiting effect on *E. coli* and *S. aureus* (Adams et al., 2014), kiwifruit pollen development (Speranza et al., 2010) and peripheral blood mononuclear cells (Petrarca et al., 2014). It is therefore necessary to evaluate risk factors of Pd-NPs as well as other metallic NPs via standardized tests before their application (Nowack and Bucheli, 2007; Wiesner et al., 2006). Particularly, the information on their impact on the active microbial community (Farré et al., 2008; Sánchez et al., 2011), which is the most important player in bioremediation processes, is missing.

Therefore, the aim of this work was to assess the ecological toxicity of bio-Pd NPs in marine environment using Microtox® standardized acute toxicity test (Ma et al., 2014) and to evaluate their impact on a PCB-dehalogenating marine microbial community in its contaminated sediments of origin. In particular, the effects on the main respiratory metabolisms of the examined microbial community (i.e., dechlorination, sulfate reduction and methanogenesis), as well as on the total bacterial community structure were assessed.

Materials and Methods

Preparation of bio-Pd NPs

Preparation of bio-Pd NPs was performed through bioprecipitation with *Shewanella oneidensis* pure cultures according to De Windt, et al. (2005).

Ecotoxicity tests on V. fischeri

Acute toxicity of bio-Pd NPs was measured by assessing luminescence inhibition of the marine Gram-negative bacterium *V. fischeri* (strain NRRL B-11177) with a Microtox® M500 (Modern Water Monitoring, Cambridge, UK) after 5, 15 and 30' exposure to serial 2-fold dilutions of a bio-Pd NPs solution at 9.5 ppm (equivalent to 50 mg/kg dry sediment condition). The luminescence signals were normalized against an active control with no bio-Pd NPs using the MicroTox Omni Software, according to the 81.9% Basic Test Protocol provided by the manufacturer. The concentration of the sample (ppm) which produces a 50% decrease in bioluminescence after exposure is designated as the effective concentration EC_{50}.

Microcosm preparation and sampling

The impact of bio-Pd NPs on a PCB-dechlorinating marine microbial community previously enriched from a sediment collected from the Venice lagoon (Italy) was tested by sub-culturing the community in anaerobic 70 ml slurry microcosms prepared as follows. First, sediment from the Venice lagoon was autoclave-sterilized at 121°C for 1 h on 3 consecutive days with incubation at 28 °C between each autoclaving treatment. 1600 ml of site water were purged in a 1 l Erlenmeyer flask with filter-sterilized N_2:CO_2 (70:30) with a Hungate-similar apparatus for 2 h under vigorous magnetic stirring. Then, degassed water was filter-sterilized and added to the autoclaved sediment to obtain a 20 % (dry w/v) suspension and the resulting slurry was mixed and purged as described above for 2 additional hours. Seventy ml aliquots of sediment slurry were then withdrawn while mixing and purging, and transferred into 120 ml serum bottles and sealed with Teflon-coated butyl stoppers under N_2:CO_2 flush. Three sets of microcosms were prepared: i) a set inoculated (5% v/v) with the PCB dechlorinating culture and spiked with the PCB mixture Aroclor 1254 (from a 20 g/l stock solution in acetone) at a final concentration of 1 g/kgdw; ii) a set inoculated (5% v/v) with the same culture but not spiked with PCBs (equal amount of PCB-free acetone added instead of the PCB stock solution); iii) a set non-

inoculated (i.e., sterile) and spiked with Aroclor 1254 at a final concentration of 1 g/kgdw. The two sets spiked with PCBs comprised the following conditions: i) not amended microcosms (biologically active and sterile controls), ii) microcosms amended with H_2:CO_2 (70:30) atmosphere, as control to investigate the effect of hydrogen, required for bio-Pd catalytic activity, on respiratory activities and the community structure (inoculated, i.e., biologically active set) and as additional negative control for PCB chemical dechlorination (not inoculated, i.e., sterile set), (iii) microcosms amended with H_2:CO_2 (70:30) atmosphere and bio-Pd NPs at 5 mg/kgdw and iv) amended with H_2:CO_2 (70:30) atmosphere and bio-Pd NPs at 50 mg/kgdw, to study the effect of different bio-Pd NPs concentrations on the community activity and structure (biologically active set) and the PCB dechlorination activity of bio-Pd NPs (sterile set). The inoculated set that was not spiked with PCBs included all conditions above except for the not amended microcosms, and was set up to confirm the effect of bio-Pd NPs on the activity and structure of the microbial community in the absence of organic contaminants. Triplicate microcosms were set up for each condition. All microcosms were incubated statically in the dark at 28°C and periodically sampled according to the procedure described in Zanaroli et al. (2012a) to analyse i) the volume and composition of the biogas produced in the microcosm headspace, ii) the type and concentrations of PCBs in the sediment, iii) the concentration of sulfates in the water phase, iv) the structure of the microbial community.

PCB extraction and analytical procedures

PCB extraction was performed in duplicate from each replicate culture. PCBs were batch extracted from 0.3 mL aliquots of sediment slurry with 3 volumes (0.9 ml) of hexane:acetone (9:1), 0.150 ml of elemental mercury and octachloronaphtalene (OCN) at final concentration of 0.4 ppm were added according to Fava et al. (2003). The qualitative and quantitative analyses of the extracted PCBs were performed with a GC-ECD under the analytical conditions described elsewhere (Fava et al., 2003a). Qualitative analysis of the freshly spiked PCBs and their possible dechlorination products was performed by comparing the retention time (relative to OCN) of the peaks obtained from the analyses of the sediment organic extracts with those of PCBs occurring in Aroclor 1242 and Aroclor 1254 PCB standard mixtures analysed under identical conditions. Quantitative analyses of each PCB congener were performed by using the GC-ECD response factor of each target PCB obtained through linear five-points calibration curves of Aroclors 1254 and 1242 (in the range 1 to 50 ppm each mixture) as described in Fava et al. (2003a). Response factors were verified monthly. PCB concentrations (μmoles/kgdw), average number of Cl per biphenyl and dechlorination rates (μmoles of Cl released per

kgdw per week) were calculated assuming co-eluting congeners to be present in equal proportions as described in previous works (Zanaroli et al., 2010).

Biogas production was measured at each sampling with an airtight glass syringe, while its composition was determined via μGC-TCD as described previously (Scoma et al., 2011). Sulfate concentration in the water phase was determined with IC-CD as described in (Fava et al., 2003a); linear 5-point calibration curves (1.0–25.0 ppm range) for SO_4^{2-}, Cl^-, NO_3^- and NO_2^- were obtained by using mixtures of these compounds.

Community analysis by PCR-DGGE of the 16S rRNA gene

Slurry samples (2 ml) were centrifuged at 10,000*g* for 10 minutes and the water phase was discarded; metagenomic DNA was extracted from the wet sediment (approximately 250 mg) recovered DNA was extracted with the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Sediment samples suspended in the bead solution supplied with the kit were treated with 4.5 μl of a 100 mg ml^{-1} Proteinase K solution from *Streptomices griseus* (Sigma-Aldrich, Milano, Italy) and 8.2 μl of a 100 mg ml^{-1} of chicken egg Lysozyme (Sigma-Aldrich, Milano, Italy) solution at 37°C under shaking at 150 rpm for 30 min, prior to cell lysis step described in the provided protocol.

For DGGE analysis, 16S rRNA genes of the bacterial community were PCR amplified from the metagenomic DNA with the GC-clamped forward primer GC-357f (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGGCAGCAG-3') and the reverse primer 907r (5'-CCGTCAATTCCTTTGAGTTT-3') (Sass et al., 2001) with PCR conditions described elsewhere (Zanaroli et al., 2012b). DGGE of bacterial amplicons (approximately 400 ng DNA per lane) were performed with a D-Code apparatus with a denaturing gradient from 40% to 60% denaturant as described in Zanaroli et al. (2010). Gels were stained with SYBR Green I (Sigma-Aldrich, Milano, Italy) and their image captured in UV transilluminator with a digital camera supported by a Gel Doc apparatus (Bio-Rad, Milan, Italy).

Community richness (Rr) and community organization (Co) indexes were calculated from DGGE image analysis as described in literature (Marzorati et al., 2008; Read et al., 2011; Wittebolle et al., 2009). In particular, the range-weighted richness was calculated from the total number of bands in the pattern and the denaturing gradient comprised between the first and the last band of the pattern, whereas the community organization was derived from Pareto-Lorenz (PL) evenness curves and the respective Gini coefficient times 100.

Results

The impact of bio-Pd NPs was assessed firstly on a model marine bacterium, *V.fischeri*, by assessing eventual inhibition of its bioluminescence. Then, tests focused on the eventual impact of bio-Pd NPs on the main respiratory metabolisms and the structure of a PCB dechlorinating marine microbial community. For this purpose, anaerobic sediment sub-cultures were set-up, in the presence and in the absence of spiked PCBs either: i) in absence of any amendments (unamended controls); ii) in presence of 70% hydrogen atmosphere, which is necessary for the catalytic activity of bio-Pd NPs; iii) in presence of 70% hydrogen atmosphere and two different concentrations of bio-Pd NPs.

Acute toxicity standard tests with V. fischeri

To assess the acute toxicity of bio-Pd NPs on the evolution of a marine microbial community, a standard test was performed by assessing of the decrease in luminance of *V. fischeri* in presence of different concentrations of nanoparticles. A slight inhibition of luminescence (12%) was detected only after 5 minutes of incubation in the presence of the highest bio-Pd NPs concentration (Fig 1). In all other cases (both in terms of bio-Pd concentration and exposure time) stimulating effects were detected, as luminescence was higher than non-amended control (percentages of inhibition below 0%). Therefore, no EC_{50} could be determined. Such a biostimulation effect might be due to the presence of some cell debris associated to the bio-Pd NPs. Therefore, bio-Pd NPs do not result to be toxic at the concentrations used in this experiment, that were previously shown to be effective in the dehalogenation of different organohalides in marine sediments (Hosseinkhani et al., 2015).

Respiratory metabolisms

The unamended control (hydrogen free and bio-Pd NPs free) showed the highest dechlorination activity among the whole PCB-spiked, biologically active microcosms: the average number of chlorine atoms per biphenyl molecule decreased from 5.1 to 4.3 in 18 weeks of incubation, leading to the depletion of 54±3% of penta- to octachlorinated congeners at the maximum rate of 243±16 μmol Cl removed $week^{-1}$ $kgdw^{-1}$ (Fig 2). The presence of hydrogen in the headspace, either alone or in combination with bio-Pd NPs, decreased remarkably the dechlorination rates, all of which were less than half of unamended microcosms (107±37, 118±75 and 97±27 μmoles of chlorine removed $kgdw^{-1}$ $week^{-1}$ for hydrogen-amended microcosms with no bio-Pd NPs, bio-Pd-NPs at 5 mg/kgdw and bio-Pd

NPs at 50 mg/kgdw, respectively). The final average number of chlorine substitution after 18 weeks of incubation resulted to be 4.7, 4.7 and 4.9, corresponding to depletion percentages of highly chlorinated congeners as low as 25%, 24% and 21%, for the hydrogen-amended microcosms with no bio-Pd NPs, bio-Pd-NPs at 5 mg/kgdw and bio-Pd NPs at 50 mg/kgdw, respectively (Fig 2). The dechlorination specificity led to a predominant accumulation of 24-25-CB and 24-34/236-25-CB (14 mol %) (Fig 3a). In addition, the high residual concentration of the main penta-chlorinated biphenyls of spiked Aroclor 1254 mixture indicates that the dechlorination process was still incomplete when the incubation stopped. Coherently with the lower activities in all hydrogen-amended microcosms, and regardless of bio-Pd NPs, lower percentages of tetra- and tri-chlorinated congeners were detected at the end of incubation with a specificity apparently not influenced by bio-Pd NPs (Fig. 3). From these observations, bio-Pd NPs do not seem to impact the microbial dechlorination activities. No dechlorination was detected in any of the sterile microcosms, regardless of hydrogen atmosphere and bio-Pd NPs amendments (Fig 2).

Regarding other respiring metabolisms, sulfate-reduction activities were marked in all biologically active microcosms: the initially occurring sulfates (2.5 g/l average) were completely consumed in the not-amended active controls after 6 weeks of incubation. A similar sulfate consumption was detected in microcosms amended with hydrogen only, while sulfate was completely consumed only after 9 weeks of incubation in microcosms supplemented with hydrogen and bio-Pd NPs at 5 mg/kgdw, and by the end of incubation in microcosms supplemented with hydrogen and 50 mg/kgdw of bio-Pd NPs (Fig 4a). Thus, a dose-depended inhibition of bio-Pd on sulfate reduction was observed. Similar dose-dependent effect of bio-Pd on sulfate reduction was also detected in the PCB-free sediment microcosms (Fig 4a).

No sulfate reduction, nor methane accumulation, were observed in the sterile microcosms throughout incubation (data not shown). In PCB-dechlorinating microcosms, hydrogen atmosphere stimulated methanogenesis compared to the not amended control, leading to a production of 27 ml of methane in the bio-Pd NPs-free microcosms versus 6 ml in the unamended control after 18 weeks on incubation (Fig 4b). In addition to the positive effect of hydrogen, however, a negative effect of bio-Pd NPs on methanogenesis was detected, since 17±1 and 13±1 ml of methane were produced respectively in the microcosms amended with 5 mg/kgdw and 50 mg/kgdw of bio-Pd NPs, corresponding to 62% and 48%, respectively, of the methane produced in the bio-Pd NPs-free control (Fig 4b). Similar dose-dependent inhibition by bio-Pd NPs on methanogenesis was observed in the microcosms without PCBs, as the methane production decreased of 44% and 59% compared to hydrogen-amended bio-Pd

NPs-free microcosms, in the presence of 5 mg/kgdw and 50 mg/kgdw bio-Pd NPs, respectively (Fig 4b).

Influence of bio-Pd NPs on the microbial community composition

To further verify the impact of bio-Pd NPs on the total microbial community, PCR-DGGE approach was used to determine variations in terms of Community organization (Co) and Richness (Rr) throughout incubation. PCR-DGGE analyses were carried out on the bacterial communities of biologically active microcosms at the beginning, at the half and at the end of incubation (0, 9 and 18 weeks, respectively) (Figs 5 and 6).

A marked increase of community richness was detected in all hydrogen-amended dechlorinating microcosms, and especially in presence of bio-Pd NPs, which exhibited a dose-dependent increase effect. The final richness (Rr) values were 25, 30, and 29 for microcosms with hydrogen only, hydrogen and bio-Pd NPs at 5 mg/kgdw and hydrogen and bio-Pd NPs at 50 mg/kgdw, respectively, while only 21 in unamended active controls (Fig 5). The community organization gradually decreased over time in the unamended controls, as the Co decreased from 40 to 32; on the contrary, all dechlorinating microcosms amended with hydrogen showed a transient decrease in evenness, followed by a successive re-organization, to reach final Co similar to values measured in the unamended dechlorinating control (Fig 5). Thus, bio-Pd NPs did not seem to exhibit any effect on the community organization of the microbial dechlorinating community, while apparently stimulating a richer microbiota.

The same analysis was also performed on microcosms without PCBs (Fig 6) revealing again a dose-dependent increase effect by bio-Pd NPs on the richness of the bacterial community at the end of the incubation. In the absence of PCBs a decrease in community organization was observed over time. This suggests that the community re-organizes, possibly because of the loss of some selective pressure exerted by PCBs and thus of the competitive advantage of the organohalides respiring members, which possibly favoured other species as the dechlorinating species decay (Fig 6). However, no differences were observed in the community organization of bio-Pd amended and bio-Pd free microcosms, indicating the bio-Pd NPs do not affect significantly the community structure.

Discussion

The use of bioPd-NPs as remediation catalyst is currently gaining a momentum, providing an effective strategy for the treatment of recalcitrant compounds such as azo dyes (Quan et al., 2015) or organohalides (Hosseinkhani et al., 2014b; Mertens et al., 2007) in several contaminated matrices, such as wastewater (Hennebel et al., 2012), groundwater (De Corte et al., 2012) and sediments (Hosseinkhani et al., 2015). NPs in general may pose risk for aquatic environments (Moore, 2006) and their delivery, either by *in situ* application or by accidental release, might result in unwanted effects which would eventually invalidate their benefits (Grieger et al., 2010; Karn et al., 2009; Schrand et al., 2010). The evaluation of the risk/benefit ratio is not trivial, due to the lack of standard experimental conditions and analytical protocols for nanoparticles in general (Crane et al., 2008; Grieger et al., 2010) which persists in spite of a huge research need, still unaddressed (US-EPA 2014, 2007). In addition, the literature regarding Pd-NPs toxic effect is quite scarce. It is known that they can exert antimicrobial effects (Adams et al., 2014) and that they might be toxic for some superior organisms (Lüderwald et al., 2016; Petrarca et al., 2014) but not for others (Kovrižnych et al., 2013; Shah and Belozerova, 2009). In this study, a standard *V*. *fischeri*-based ecotoxicity assay has been implemented, being currently referred as a standard test also for NPs toxicity (Sánchez et al., 2011). The test is more sensitive than other standard tests reported, such as *D*. *magna* toxicity (van Beelen, 2003), highly specific and recommended to investigate the toxicity of various metals. To the best of our knowledge, this is the first study in which such assay was used on Pd-NPs and the obtained results are dismissing hypotheses of toxic effect of this these NPs on marine bacteria.

Some limitations characterize standard ecotoxicity assays since they usually miss the interplay between different factors, affecting both the environmental fate of the delivered/dispersed nanoparticles and their interaction with the pollutant and the organisms (Nowack and Bucheli, 2007). To the best of our knowledge, no information on the toxicity of Pd-NPs over environmental microbial communities is available. Moreover, the effect of the natural organic matter, which occurs in complex environments such as aquatic sediments, should not be underestimated: it is well known that organic matter not only can change the catalytic properties of metal NPs, but can also affect exposure and toxicity to (micro)organisms (Wang et al., 2016). In addition, the bio-Pd NPs used for this study are embedded in the cell wall of killed *S*. *oneidensis* and this might influence their toxicity to bacteria, by limiting their entry into the cells or altering their interaction with the cell surface and the sediment organic matter; on the other hand, the cell lysis might generate free bio-Pd NPs over time, thus changing their potential impact. Therefore, prior actual delivery or accidental leaks into the environment, and regardless of their catalytic efficiency, bio-Pd NPs environmental impacts need to be assessed, i.e. on microbial activity

342 and on microbial community structure. For all of these reasons, thorough tests were required in
343 matrices close to the environmental conditions and for longer incubation time than current standard
344 tests. Microcosms studies are a good compromise between the complexity of environmental matrices
345 and the need to get preliminary data on the impact of NPs over microbial communities (Echavarri-
346 Bravo et al., 2015), which then need be thoroughly investigated further in mesocosms (Holden et al.,
347 2016). Therefore, tests were performed in microcosms of actual marine sediments and water,
348 inoculated with a known dehalogenating microbial community in biogeochemical conditions close to
349 those occurring in the real site: this allowed to evaluate both the catalytic activity and the impact of
350 bio-Pd NPs on the microbial community under environmental conditions and in a time frame suitable
351 for slow-growing anaerobic microorganisms. A culture enriched in organohalide respiring members
352 was selected because bio-Pd NPs could be used to complement biological reductive dechlorination
353 activities with catalytic dehalogenation in nanoremediation approaches.
354 In this study, a lack of catalytic activity by bioPd-NPs over PCBs was evidenced in the sterile sediment
355 microcosms. A similar lack of activity was already shown to occur for bioPd-NPs in another anoxic
356 sediment of the Venice Lagoon area, where it was due to some inhibiting effects of the environmental
357 matrix, i.e., sulfide-poisoning phenomena (Hosseinkhani et al., 2015). This further supports the
358 assumption that bio-Pd NPs are effective catalysts in low-sulfidogenic sediments, i.e., in presence of
359 low sulfate concentration or under more aerobic conditions (Hosseinkhani et al., 2015). The complex
360 matrix of sediments might as well have played an inhibiting role, since the environmental distribution,
361 fate and activity of many NPs could be influenced by phenomena of sequestration, adsorption and
362 chemical inactivation by humic acids (Shah and Belozerova, 2009; Wang et al., 2016). Further studies
363 could be performed on different sediments not affecting the catalytic activity of bioPd-NPs to exclude
364 their toxic effect on microbiota in effectively catalytic conditions; however, inactivation of bio-Pd NPs
365 is a possible occurrence in the actual sediment used in this study, making relevant the evaluation of
366 bio-Pd NPs impact on microbial communities independently from their activity.
367 A negligible effect of bio-Pd NPs was observed on organohalide respiration. It has been shown that
368 other zero-valent metal NPs, known to be effective enhancers of PCB reductive dechlorination
369 activities in marine sediments, such as zero-valent iron NPs (nZVI), (Zanaroli et al., 2012b) do exhibit
370 also a series of ecotoxic effects in the environment, recently reviewed extensively by Lefevre, et al.
371 (2015), which include severe impacts on organohalides respiring communities in groundwater (Barnes
372 et al., 2010) and soil (Tilston et al., 2013), either through shifts in the microbial composition (Tilston et
373 al., 2013) or direct inhibition (Z. M. Xiu et al., 2010). A dose-dependent inhibitory effect exerted by

bioPd-NPs was evident on sulfate reduction and methanogenic activities. Literature data on the effect exerted on these respiratory metabolisms in anaerobic microbial communities by Pd-NPs or similar nanoparticles, such as nZVI, are limited and often contradictory: either inhibition of sulfate reduction activities (Barnes et al., 2010) or increase in sulfate reducing bacteria population (Kirschling et al., 2010), either increase in methane production (Hu et al., 2015) or its inhibition (Huang et al., 2016) were reported. Studies investigating the mechanisms of interaction between bio-Pd NPs and cells of different microbial species might be useful to understand why bio-Pd NPs exert an inhibitory effect on some respiratory metabolisms rather than others.

Microbial community analysis is a fundamental tool to investigate toxicity hypotheses on environmental microbial communities (Sánchez et al., 2011). Particularly, diversity highly influences microbial community efficiency (Tilman et al., 2014), but also evenness plays an important role under selective stress (Wittebolle et al., 2009). It would be expected that, in presence of a toxic compound, both indexes would significantly decrease in a marine community (Johnston and Roberts, 2009), due to a selective growth of the resistant community members. BioPd-NPs, instead, only slightly altered the structure of the microbial community. In addition, they had a clear positive effect on community richness, while causing only a temporary shift in its community organization, which was reversed at the end of incubation. These observations clearly indicate that bioPd-NPs have no significant effect of on the overall microbial community. In other studies, more remarkable changes in the microbial community composition with nZVI NPs were reported, which were interpreted as a toxic effect (Kirschling et al., 2010; Tilston et al., 2013; Z. ming Xiu et al., 2010); however, in these cases no analysis of the community richness and community organization was performed, not allowing a clear comparison of the overall effects of bioPd-NPs and nZVI-NPs on microbial communities. Also, the different catalytic mechanisms of these two NPs (nZVI are reagents, while Pd-NPs are catalysts) may explain the differences in the toxic behaviours, as the peculiar microbial community structure and the interplay among environmental factors and NPs can do (Lefevre et al., 2015). Finally, microbial communities in marine sediments often demonstrate the capacity to resist to perturbations; even when ecological shifts are observed, particular metabolic functions of the community might be uncoupled, making the community eventually resilient to external forces (Bowen et al., 2011). The marine sediment community used in this study showed resiliency to bio-Pd NPs. Although this property might mask possible toxic effects on some specific members of the community, combined with the lack of toxicity to *V. fisheri* and the limited influence on few respiration activities observed, this information further supports the lack of significant impacts of bio-Pd NPs on marine bacteria. Overall, these data

406 also point out the importance of combining multiple tests addressing different types of effects (standard
407 ecotoxicity assays, monitoring of metabolic activities and community structure) to evaluate the impact
408 of NPs on the environmental microbiota.
409
410 **Conclusions**

411 Different approaches have been used to evaluate the effect of bio-Pd NPs on marine microbes and
412 communities, taking into consideration their main metabolic activities, their biodiversity and
413 community structure. Bio-Pd NPs do not exert toxicity towards the bioluminescent marine bacterium
414 *V. fisheri*. They may have limited inhibitory effects selectively towards specific respiratory
415 metabolisms, such as sulfate reduction and methanogenesis, but not organohalide respiration. On the
416 other hand, an increase of the community biodiversity along with no permanent effects on its
417 community organization have been observed, indicating the lack of a significant impact on the
418 microbial community. Overall, these data combined dismiss hypotheses of ecological impact of bio-Pd
419 NPs on marine microbiota. The use of a combination of different approaches tailoring as many factors
420 as possible should be implemented when investigating the possible impact of NPs in the environment.
421
422 **Acknowledgements**

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424
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Figure Captions

Figure 1. Bioluminescence inhibition of *V. fischeri* in acute toxicity tests after 5, 15 and 30 minutes exposure to different dilutions of bio-Pd NPs in marine filter-sterilized water. The highest concentration of bio-Pd NPs corresponds to 81.9% of the 50 mg/kgdw concentration at which bio-Pd NPs were applied in the microcosms experiment. Inhibition is calculated in percentage against control with no bio-Pd NPs. Values are means of duplicate analysis.

Figure 2. Dechlorination of Aroclor 1254 PCBs over time in the biologically active (inoculated) and sterile (non inoculated) sets of spiked microcosms. Values are means of triplicate microcosms, with error bars representing standard deviation.

Figure 3. Concentrations of spiked PCB congeners and their dechlorination products constituting more than 1% w/w of total PCBs at the end of incubation in the biologically active (white bars) and sterile (black bars) sets of spiked microcosms. (a) unamended microcosms; (b) hydrogen-amended microcosms; (c) microcosms amended with hydrogen + bioPd 5mg/kgdw; (d) microcosms amended with hydrogen + bio-Pd 50 mg/kgdw. Values are means of triplicate microcosms with error bars representing standard deviation.

Figure 4. Sulfate concentration (a) and methane production (b) in the biologically active sets of microcosms spiked and not spiked (No PCB) with Aroclor 1254. Values are means of triplicate microcosms with error bars representing standard deviation.

Figure 5. Upper panel: DGGE profiles of the total bacterial community in the biologically active, PCB-spiked set of microcosms at weeks 0, 9 and 18. In each lane, PCR products obtained from the three replicate microcosms were pooled. Lower panel: analysis of the Community organization (Co expressed as Gini percentage times 100, bars) and richness (diamonds) for each DGGE lane.

Figure 6. Upper panel: DGGE profiles of the total bacterial community in the biologically active, PCB-free set of microcosms at weeks 0, 9 and 18. In each lane, PCR products obtained from the three replicate microcosms were pooled. Lower panel: analysis of the Community organization (Co expressed as Gini percentage times 100, bars) and richness (diamonds) for each DGGE lane.

Figure 1

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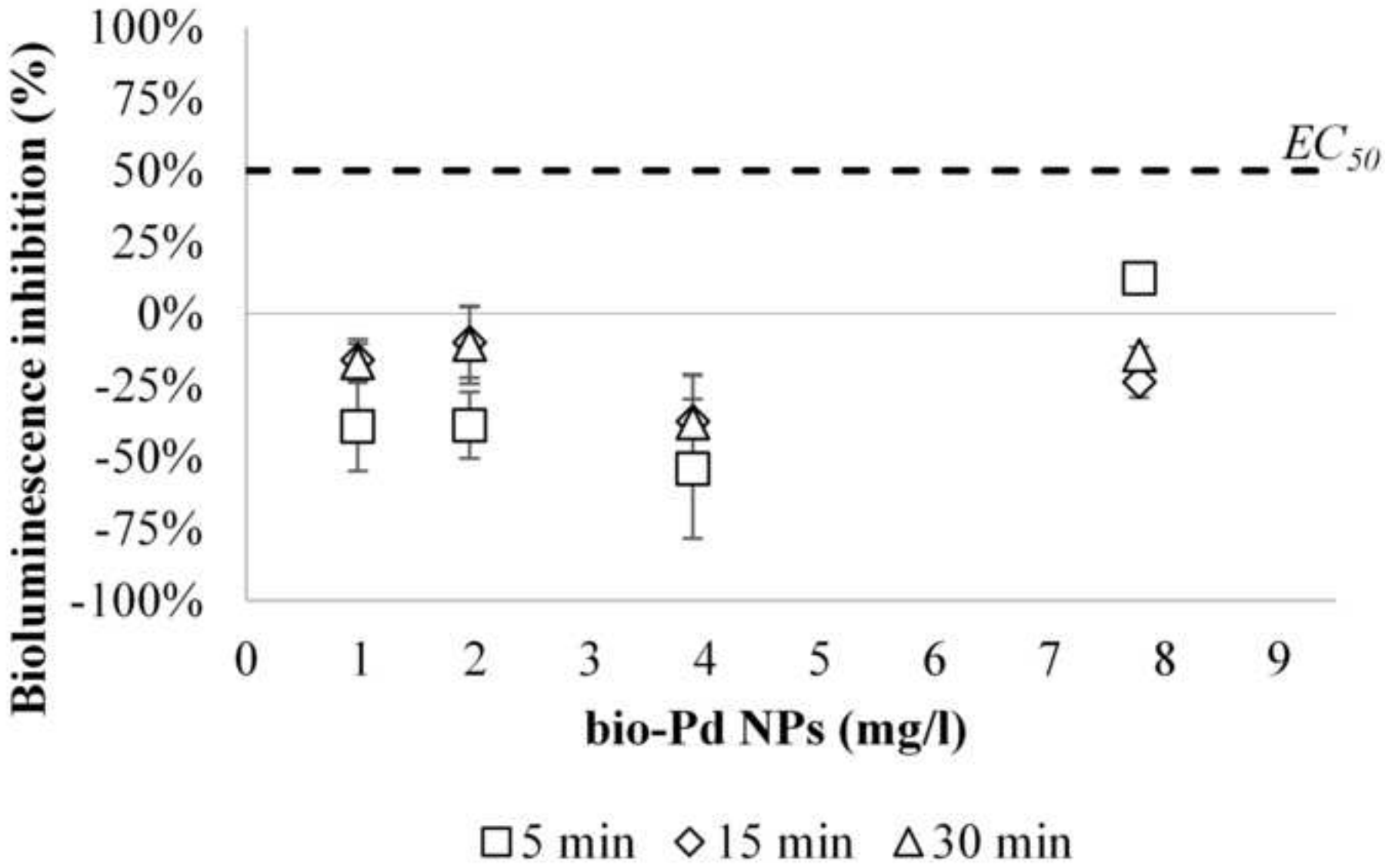

Figure 2

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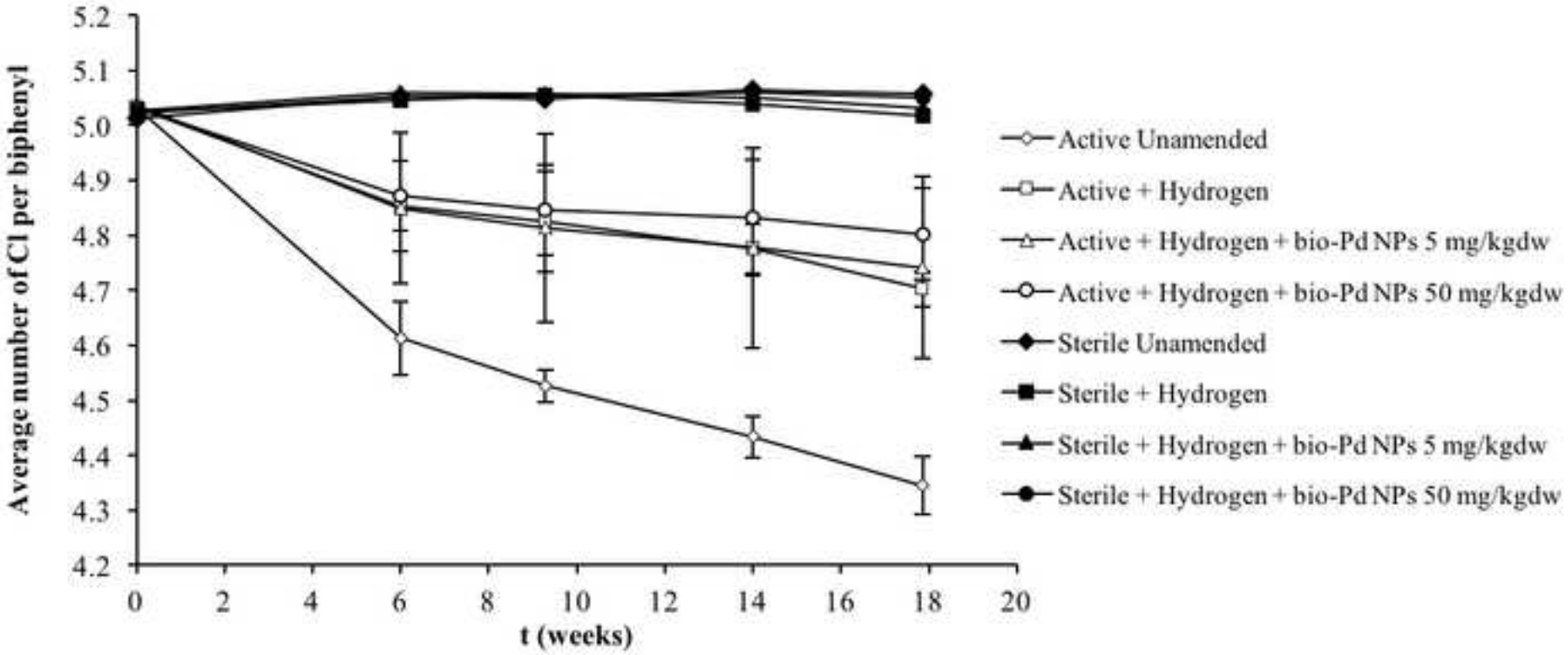

Figure 3

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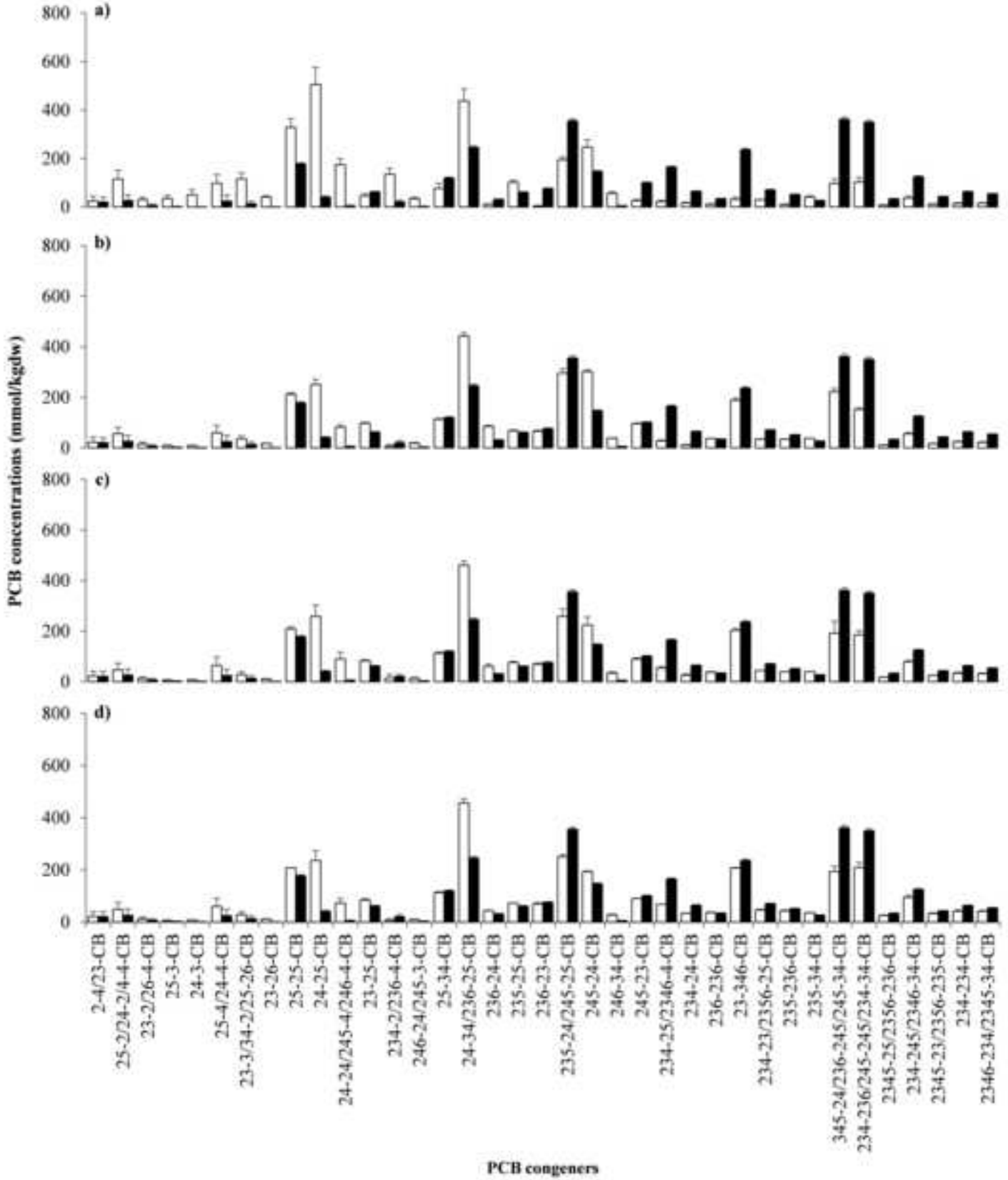

Figure 4

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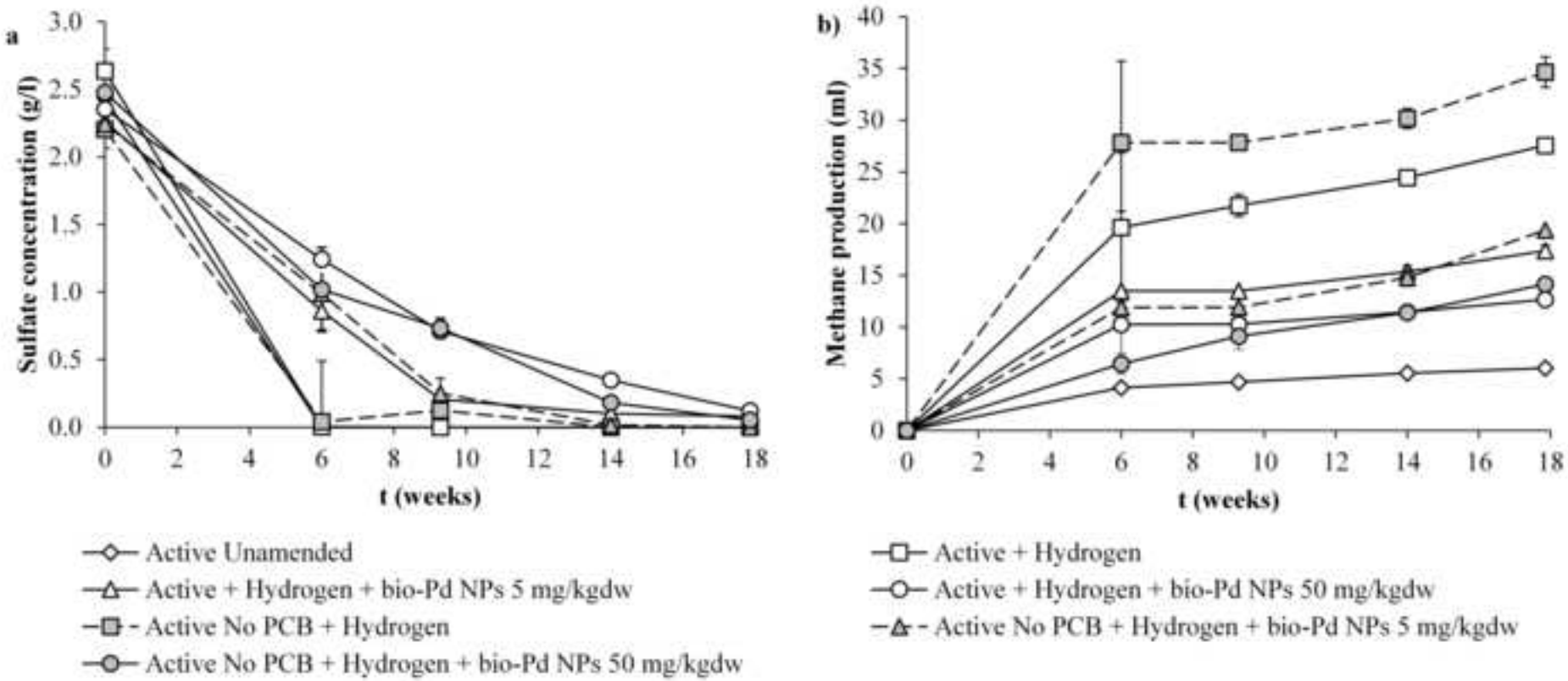

Figure 5

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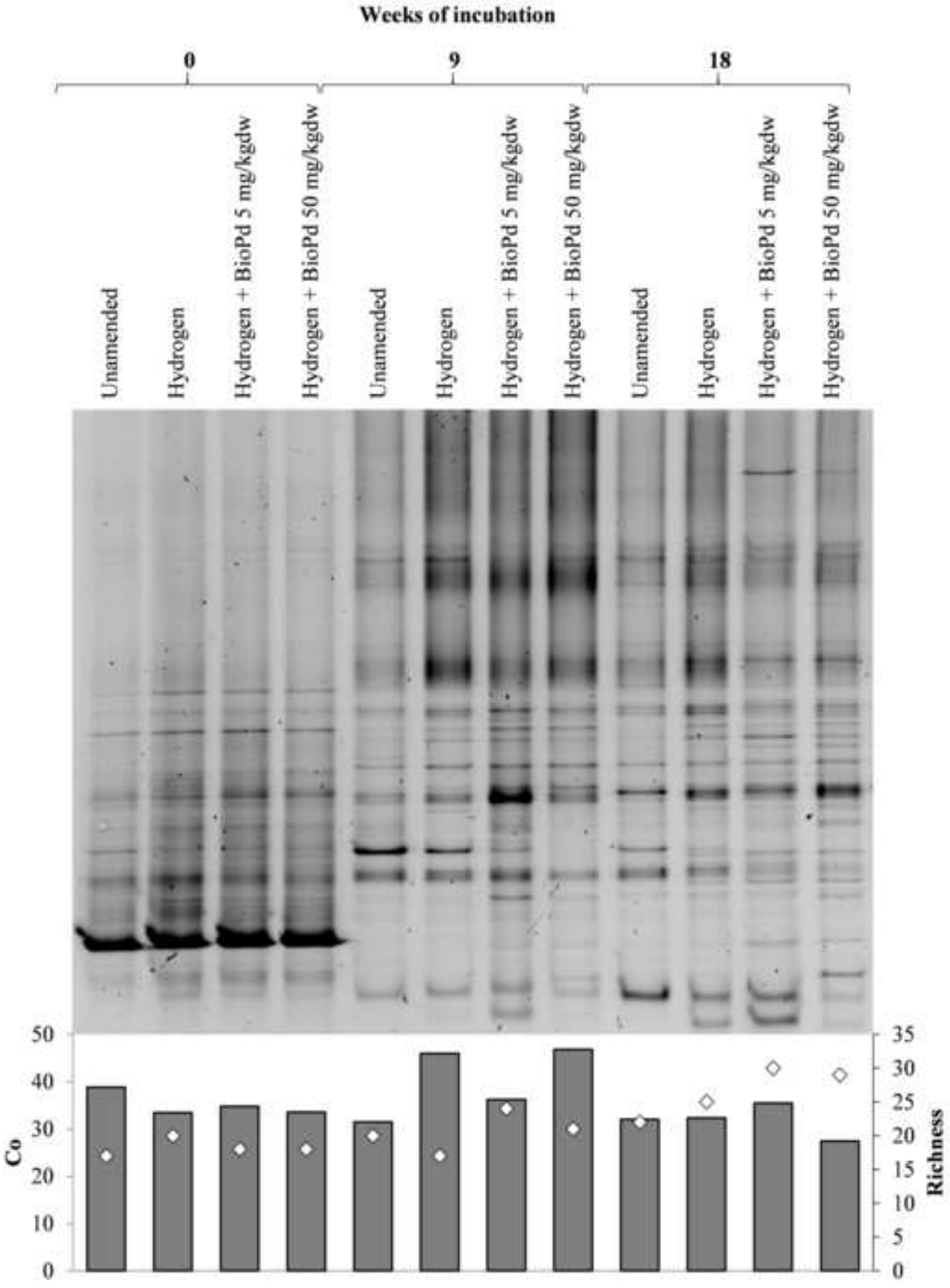

Figure 6

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