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The influence of polystyrene nanoparticles on enzyme clusters of fumarate, malate dehydrogenase and citrate synthase: a fractal analysis study

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ABSTRACT

In the context that plastic debris could degrade into smaller and smaller particles especially at the nanoscale, concerns about their ecotoxicological effects are raised. The purpose of this study was to determine the biophysical effects of neutral polystyrene nanoparticles (NPs) on the activity of fumarase-malate dehydrogenase (MDH)-citrate synthase (CS) cluster both *in vitro* and in mussels exposed to NPs. At first, commercial preparations of fumarase, MDH, CS were examined in the presence of NPs to determine changes in the fractal environment based on time dependence MDH and CS rates and changes in the spectral dimension (sD) of the cluster. In second part, these effects were also examined in mussels exposed to either polystyrene NPs or in mussels exposed to municipal effluents, which are suspected environmental sources of plastic NPs. The results showed that the presence of NPs decreased the time dependence of MDH rate and increased significantly the sD. A sD=0.55 of the *in vitro* reaction revealed a close organization of the enzyme cluster, which was equivalent to a random percolation cluster with 74% of space occupancy. The addition of 50 and 100 nm in the reaction media reduced the space occupancy to 50 and 33% respectively. Exposure of mussels to 50 nm NPs for 24h lead to increased levels of NPs in the digestive gland and revealed similar effects in the digestive gland subcellular fraction where the sD was increased from 1.25 to 1.48 at the highest concentration of 5 mg/L reducing the space occupancy of 35% compared to 46% in control mussels. Exposure of mussels to a municipal effluent for 3 months also lead to increased polystyrene-like NPs in the digestive gland and increased fumarase-MDH-CS activity and the sD even further to 23% space occupancy. In conclusion, polystyrene NPs have the capacity to increase the sD of the cluster decreasing the normal time-dependence of enzyme activity of the fumarase-MDH-CS cluster thereby maintaining energy metabolism for longer times which could deplete energy reserves.

Keywords: *fumarase; malate dehydrogenase; citrate synthase; clusters; fractals; spectral dimension; plastic nanoparticles.*

1. INTRODUCTION

The widespread distribution of plastic debris in many aquatic habitats has raised the issue of their potential ecotoxicity. Plastics have been detected nearly in most habitats i.e., in the water column, sediments and biota [1] making them one of the major contamination problem of the 21st century. Costal waters and sediments contained in the order of 7 microplastic particles/m^3 and 5 microplastic particles/g respectively. Plastic debris was found in both anchovies and oysters, which contained between 3-8 microplastic particles/individuals. The study also showed that microplastics are released from solid waste disposal sites and municipal effluents. Plastic debris could degrade in the environment forming ever-fine particles down at the micron and nanometer ranges. For example, on plastic tea bag was shown to release 3.5 billion plastic nanoparticles after only single use at brewing temperature of 95°C [2]. Particles at the nanoscale have the potential to permeate cells and reach the cytoplasm where many biochemical processes are at play. The detection of NPs in tissues is more difficult to determine owing to the lack of methods to detect them at this scale. However, a recent study revealed that mussels exposed to municipal effluents had elevated levels polystyrene-like NPs based on a fluorescence polarization assay for polystyrene NPs [3]. Although plastic NPs are considered as relatively inert, it is anticipated that the accumulation of plastic NPs in cells will increase the crowding environment and perhaps disrupt the spatial organization of proteins/enzyme network in the intracellular compartement. Means to determine the biophysical changes of the space domain of enzymes NPs in tissues and their long-term consequences are urgently needed to support hazard assessment of plastic contamination.

Enzymes were shown to associate with each other and structural proteins (cytoskeleton) to form highly efficient "functional units" sometimes termed metabolons or fluxons [4, 5]. These units enable cells to maintain high activity at low substrate levels in situations of limited substrates (e.g., fasting), which are ways for cells to regulate metabolism. Enzymes have the ability to form temporary dissipative structures, which can be isolated by gentle separation techniques such as gel filtration or density gradient centrifugation. Enzymes involved in the tricarboxylic acid (TCA) cycle are central for aerobic metabolism in both prokaryotes and in the mitochondrial matrix of eukaryotes. For example, the following enzymes were shown to form such clusters: fumarase, malate dehydrogenase (MDH), citrate synthase (CS), aconitase and isocitrate dehydrogenase. These complexes are fragile and could easily dissociate by crowding effects by other large molecules such as NPs. Functional units of fumarase-MDH-CS were isolated by gentle technics such as gel filtration [6]. It was shown that 4 MDH was associated with one fumarase and one CS enzyme forming a 6 units cluster. The cluster involves the sequential transformation of fumarate to citrate, isocitrate and α-cetoglutarate [4] where the close association between these enzymes permits channelling of the intermediates in a restricted space for optimal activity in the fractal environment of the cytoplasm. Complexes of fumarase, MDH,

aconitase, CS and isocitrate dehydrogenase were shown to precipitate at 32 000 x g after 30 min [7] suggesting that these structures are relatively large with densities between mitochondria and microsomes in the size range of 100 to 200 nm. The presence of polystyrene NPs could alter hydrophobic and electrostatic interactions of biopolymers and could lead to diverse macroscopic morphologies of protein networks [8]. The changes in these morphologies could influence the local geometries and the occupational probabilities of biochemical sites, hence change the space properties of these multi-enzyme clusters.

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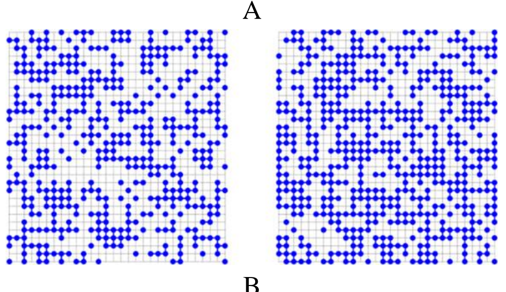

B

Percolation clusters with different crowding

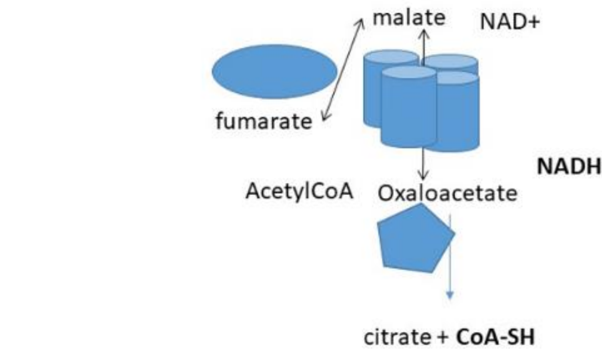

Figure 1. Percolation cluster and the fumarase, malate dehydrogenase and citrate synthase cluster.
Random percolation clusters of different crowding are shown (A). Fumarase-MDH-Cs enzyme cluster (B) are known in forming operational aggregates for optimal enzyme activity. Four MDH enzymes bind to one fumarase and CS enzyme molecules [6]. The substrate was fumarate, NAD^+ and acetylCoA yielding the products citrate, NADH and CoA-SH. **NADH** and **CoA-SH** were determined in time for MDH and CS respectively.

In the classical view of enzyme kinetics, enzyme activity is determined in steady state conditions with saturating amounts of substrate to maintain maximal rates in agreement with the mass action principle. The derivation of reaction rates and enzyme affinity (K_M and V_{Max}) parameters are considered "constants" in these conditions. Although this could be observed in simple solutions dominated by free diffusion, they do not reflect their behavior in crowded/complex environments found in cells. In crowded environments, the cellular proteins and enzymes are organized in a fractal manner where the intermediates will "percolate" through a complex maze of lattices of protein and enzyme networks before reaching the reaction center of enzymes [9]. This fractal space organization will increase the influence of time on the kinetic "constants" (Michaelis-Menten equilibrium and maximal velocity parameters), usually decreasing them in time with a significant slope h [10]. The term fractal indicates that the distribution of space occurs at different scales, which resembles a percolation structure (Figure 1A). In a fractal environment, the enzyme rates are increased at low substrate concentrations but decreased in saturating amounts of substrates. The maximal reaction rates also decrease in time following a power law rule: $k \sim k_0 t^{-h}$ where k_0 is the rate of reaction in the classical view (constant in time) and h is rate of change of k in time (slope). The slope (h) of enzyme reaction rate over time on a log-log scale is related to the spectral dimension sD by the following $h = 1-(sD/2)$. In unrestricted space, the slope is non-significant (h=0) and the sD corresponds to the Euclidian space of 2 and $k=k_0$ i.e., the rate is time invariant as found in the classical case of uncrowded environments. In a random fractal of a percolation cluster (Figure 1A) with 59% of the space is occupied, the available dimension is reduced from 2 to 1.33 with h=0.33, hence the "constants" are no longer time invariant. In other words, when the space (volume) near the substrates and the enzyme (reaction center) is reduced, the substrate and product concentrations are locally increased causing traffic hindering enzyme velocity over time. The sD is, therefore, a measure of the "space filling capacity" associated to the random walker i.e., the number of times N(t) of a walker (substrate) to randomly return to its origin (enzyme reactive center) and is expressed as: $N(t) \sim t^{-sD/2} \sim 1/t^{sD/2}$. Thus, if the sD is reduced, it will take less time for the walker to reach its point of departure thereby increasing the number of times the enzyme is visited by the substrate N(t). Conversely, if the sD is increased, it will take more time to the substrates to reach the enzyme, hence decreased number of visits of the substrate to the enzyme. However, an upper limit in the sD exists where time dependence becomes non significant as h tends to 0. This approach was developed for bimolecular reactions with equal molar amounts of substrates but could be extended to other situations and provides a means to determine the influence of molecular crowding of space where enzyme reactions take place. In fact, when the dimension of the system is reduced or "fractalized", two opposing processes are at play: the reduced dimension increases the apparent concentration of the substrate near the enzyme but at the same time the substrate could take more time to reach the enzyme in a crowded environment. It was previously shown that at low substrate concentration, the reaction rates will increase as the dimensions of the reaction are reduced but as the concentration increases, the reaction rates will tend to decrease in time [11]. In the case of multiple enzyme clusters, it is anticipated that a tight spatial organization of the enzyme clusters will increase the time dependence of the reaction rates (h), which is equivalent in reducing the sD (less free space). In situations when exogenous molecules such as plastic nanoparticles (NPs) pervade the space domain of biochemical reactions, NPs could either contribute to the molecular crowding in cells and reduce further the sD or disrupt the morphologies of enzyme clusters increasing the sD closer to unrestricted space of free space of dimension 2.

The purpose of this study was therefore to examine the influence of polystyrene NPs on the muti-enzyme complex of fumarase-MDH-CS by following the time dependence of reaction rates and the sD. These effects were first examined *in vitro* with commercial preparations of enzymes spiked with plastic NPs and then in mussels exposed for 24h to NPs suspensions. This approach

was also tested with caged mussels placed downstream a major municipal effluent dispersion plume, which is suspected sources of plastic NPs [12].

2. MATERIALS AND METHODS

2.1. *In vitro* fumarate, malate dehydrogenase and citrate synthase system.

The sequential multienzymatic system consisted of three enzymes involved in the transformation of fumurate to malate to oxaloacetate to citrate [6]. The enzymes consisted of fumarase, malate dehydrogenase (MDH) and citrate synthase (CS) involved in the following sequential reaction (Figure 1B): fumarate ←→ malate (fumarase); 2) Malate + NAD^+ ←→ oxaloacetate + NADH (malate dehydrogenase, MDH); 3) oxaloacetate + AcetylCoA→citrate + CoA-SH (citrate synthase, CS). It was shown that 4 MDH molecules were associated with one fumarase and CS molecule forming a cluster of six units (Figure 1B). The first 2 enzymes involves reversible reactions while the last enzyme (CS) is involved in an irreversible reaction. The enzymes were purchased from Sigma Chemical Company (On, Canada) and were of mitochondrial origins (i.e., from animals). The reaction media contained 10 units/mL of each enzymes in the presence of equimolar amounts (0.5 mM) of fumarate, NAD^+ and acetylCoA in 140 mM NaCl, 10 mM Hepes-NaOH, pH 7.4, 5 mM KH_2PO_4, 0.1 mM $MgCl_2$ and 10 % glycerol to mimic the density of the mitochondrial matrix. The reaction was started with the addition of the substrate mix: fumarate, NAD^+ and AcetylCo in two separated 100 µL wells. In the first sample, the formation of NADH (fumarate-MDH) was measured by fluorescence and in the second sample, the formation of CoA-SH (CS) was determined in the presence of 0.2 mM of dithiobis-nitrobenzoate in the reaction mix and the absorbance (412 nm). Readings were taken at each min for NADH and each 5 min for 60 min for CS by fluorescence and absorbance respectively. The levels of NADH were determined by fluorescence at 450 nm emission and 350 nm excitation in dark microplates (Synergy-4, Biotek Instruments, USA). The rate of increase in NADH was expressed as relative fluorescence units/min. The activity of CS was determined by the detection of CoA-SH by the addition of 0.2 mM of dithiobis-nitrobenzoate in the reaction mix and the absorbance at 412 nm in clear 96-well microplates (Synergy-4, Biotek Instruments, USA). The rate of increase in reduced thiols was expressed as the absorbance increase at 412 nm/min. Enzyme activities were determined in the presence of 50 and 100 nm polystyrene NPs (Polyscience, USA) at the following concentration: 0.0125, 0.025, 0,037 and 0.05 µg/mL. The 50 nm NPs contain 8 times more particles compared to the 100 nm NPs on a mass basis where the 50 and 100 nm NPs at 1 µg/mL contain 0.26×10^9 and 0.032×10^8 particles/mL respectively.

2.2. Exposure of mussels to polystyrene NPs.

Wild *Elliptio complanata* mussels were collected in a pristine lake in June of 2018 and brought back to the laboratory at 4°C in ice boxes. The mussels (100 individuals) were placed in 300 L aquarium maintained at 15°C under constant aeration. The aquarium water consisted of UV-treated and dechlorinated drinking water in the city of Montreal. The mussels were fed each day with commercial phytoplankton (Phytoplex) suspensions. Mussels (N=4) were placed in 4 L containers and exposed to increasing concentrations of polystyrene NPs: 0, 0.1, 0.5, 1 and 5 mg/L for 24h at 15°C. At the end of the exposure period, the mussels were placed in clean aquarium water overnight to allow purging of the gut contents and removal of adsorbed NPs. The digestive gland was dissected out of the soft tissues and weighted on ice. The digestive glandwas homogenized using a Polytron tissue grinder in 100 mM NaCl containing 25 mM HEPES-NaOH, pH 7.4, 0.1 mM dithiothreitol and 1 µg/mL apoprotinin. Preliminary experiments revealed that Polytron homogenisation disrupts mitochondrial membranes releasing the mitochondrial matrix containing fumarase, MDH and CS in the media. The homogenate was centrifuged at 1 500 x g and the supernatant centrifuged at 12 000 x g for 30 min at 2°C. The supernatant (S_{12} fraction) was kept aside and stored at -85°C until analysis. Total proteins in the homogenate and S_{12} fraction were determined using the method of Bradford (1976) [13]. Standard solutions of serum bovine albumin were prepared for calibration. The levels of polystyrene nanoplastics (NPs) in the digestive gland fraction were determined using the molecular rotor probe 9-(dicyanovinyl)julolidine (DCVJ) as previously described [12]. The DCVJ probe shows a characteristic fluorescence at 620 nm at 450 nm excitation in the presence of polystyrene NPs. Standards solutions of 50 and 100 nm polystyrene NPs (0.1-0.5 µg/mL) were used for calibration. The activity of the fumarase-MDH-CS was determined by adding the substrates mix (0.5 mM each of fumarate, NAD^+ and acetylCoA) in the S12 fraction and the formation of NADH and CoA-SH were determined as described above.

2.3. Exposure of mussels to a municipal effluent and combined sewer overflows.

The mussels were also exposed to a municipal effluent dispersion plume in the Saint-Lawrence River which are suspected sources of microplastics and nanoplastics [1, 3]. Mussels (N=20) were placed in three cylindrical (60 cm long x 15 cm diameter) cages (1 cm mesh size) and each cages placed at three sites in the St Lawrence River. The mussels (n=20) were placed at the upstream site (UPS) of a municipal effluent discharge site of the city of Montreal (2 million inhabitants), one site that receives rainfall overflows (OVF) and one site at 8 km downstream (DOWNS) the discharge site. The mussels were exposed for 2 months (23rd July to 25th September). At the end of the exposure time, the mussels were brought back to the laboratory and allowed to stand in clean aquarium water overnight to allow depuration. The mussels were then placed on ice and the digestive gland dissected and weighted. The digestive gland was then homogenized in 100 mM NaCl containing 25 mM HEPES-NaOH, pH 7.4, 0.1 mM dithiothreitol and 1 ug/mL apoprotinin using a Polytron tissue grinder (30 sec at half the maximal intensity) on ice to prevent heating. A portion of the homogenate was centrifuged at 1500 x g for 15 min at 2°C and the resulting supernatant centrifuged at 12 000 x g for 30 min at 2°C. The supernatant (S12 fraction) was kept aside and stored at -85°C until analysis. Total proteins in the homogenate and S12 fraction were determined as described above. The levels of polystyrene nanoplastics (NPs) were determined using the molecular rotor probe as described previously [12]. The DCVJ

probe show a characteristic fluorescence at 620 nm at 450 nm excitation which differed from the normal fluorescence for viscosity at 520 nm. Standards solutions of polystyrene nanoplastics (0.1-0.5 µg/mL) were used for calibration. The activity of the fumarase-MDH-CS was determined by adding the substrates mix (0.5 mM each of fumarate, NAD^+ and acetylCoA) in the S12 fraction and the formation of NADH and CoA-SH were determined as described above.

2.4. Data analysis.

In this study, the influence of polystyrene NPs on the multienzyme complex fumarase-MDH-CS was examined at different levels of environmental realism: 1) *in vitro*, 2) in mussels exposed to NPs and 3) in mussels exposed to suspected sources of NPs from municipal effluents. Enzyme activity measurements were made in duplicate and the in vitro and the laboratory NPs exposure experiments were repeated 3 times. The spectral dimension sD was calculated from the following relationship: sD=2-h where h is the slope on the log rate versus log time plot. The slopes were determined by linear regression using the least square methodology. Significance (p<0.050 was determined by rank analysis of variance and the Conover-Inman *post hoc* test. All tests were performed using the SYSTAT software package (version 13.2).

3. RESULTS

The activity of fumarate-MDH and CS was examined using the classical approach where the initial rates of reaction are obtained during the initial linear portion of product formation over time (10 min). The rate of formation of NADH/min in the presence of increasing concentrations of polystyrene NPs is presented in Figures 2A and 2B. The data revealed that the reaction rates were somewhat increased by the NPs at 0.025 and 0.037 µg/mL for the 50 and 100 nm NPs respectively. On a particle basis, it takes circa 5 times less of the 100 nm NPs to produce these changes than the 50 nm NPs. The activities were no longer increased at the highest concentration of each NPs size. The reaction rates were determined at increasing time interval and plotted in log-log plot (Figure 3A). The reaction rate for fumarate-MDH reaction decreased over time in controls with a significant slope h, which suggests time dependence in the reaction rate and changes in the sD of the enzymes cluster. A significant and negative slope in the decrease in the reaction rate in time with h=0.727 was obtained indicating a time dependence of the reaction rate of this six enzymes cluster (Figure 3A). The calculated sD (h=1-sD/2) was at 0.58 confirming the tight arrangement of this six enzyme units cluster compared to a random percolation cluster of sD=1.33 with 59% of site occupied i.e., not available for substrate movement. In the presence of 0.05 µg/mL of NPs, the slope was decreased to h=-0.40 and -0.20 for 50 and 100 nm NPs respectively. The decrease in the slopes suggests that the sD was influenced by the polystyrene NPs with the 100 nm having a stronger effect than the 50 nm NPs (Figure 3B). In controls, the sD was at 0.58 which highlights the tight organization of fumarase-MDH-CS cluster compared to 1.33 of a random percolation structure with 59 % space occupancy. Based on the relationship (sD and % of space occupancy in random percolation clusters), the multi-enzyme complex corresponds to a percolation structure with 74% space occupancy. The addition of NPs in the reaction media increased the sD in a concentration dependent manner with the 50 nm NPs producing more important changes than the 100 nm NPs. The sD increases from 0.58 in controls up to 1.15 and 1.6 for the 50 nm and 100 nm NPs respectively. This suggests that the morphological structure of the enzymes cluster is "swelled" in space in the presence of polystyrene NPs. The 50 nm NPs induces a percolation lattice with only 50% of space occupancy while the 100 nm NPs corresponds to a percolation lattice of 33% space occupancy. It was reported that a membrane pore (tube like space) had values of h=0.5 with a sD =1 corresponding to a percolation cluster with 57% occupancy [14]. This value is close to the 50 nm NPs at 0.05 µg/mL (13×10^6 particles/mL) and to the 100 nm NPs but at a lower concentration of 0.037 µg/mL (1.2×10^5 particles/mL). Hence, it takes 10-11 times less 100 nm NPs to produce a change in the sD of the fumarase-MDH-CS cluster than the 50 nm NPs.

Exposure of mussels to the 50 nm polystyrene NPs for 24 h led to the accumulation of NP in the digestive gland extract (Figure 4A). NPs were detected for all exposure concentrations (0.1-5 mg/L) in the digestive gland using the recently developed molecular rotor probe methodology for polystyrene NPs [12]. The activity of the fumarase-MDH-CS cluster was significantly increased at the highest exposure concentration (Figure 4B) indicating that NPs could influence the activity of cluster. Close examination of the time dependence in the rate of formation of NADH in time (fumarase-MDH) revealed a decreased influence of time (slope) in the reaction rates in the presence of NPs (Figure 4 C) which suggests an increase in the sD (Figure 4D). This suggests that the cluster was less tightly packed in space in the presence of polystyrene NPs compared to controls. In control mussels, the calculated sD was 1.23 corresponding to percolation cluster of 47% space occupancy. This suggests that the enzyme cluster interacts with other cytoplasmic proteins (cytoskeleton) producing a much less compact structure than the *in vitro* situation. The sD of the digestive gland fraction was increased to sD of 1.48 for the highest exposure concentration of NPs (5 mg/L) giving an equivalent percolation structure of 35% space occupancy. This suggests that exposure to NPs disrupts further the morphology of fumarase-MDH-CS cluster in exposed organisms. The presence of polystyrene NPs and effects on the fumarase-MDH-CS cluster were also studied in mussels exposed for 3 months to municipal effluents and combined sewer overflows (Figure 5). Municipal effluents are suspected source of microplastics where concentrations of microplastics were highly variable ranging from no detection to 65 particles L^{-1} [15]. The study also revealed that the size distribution of microplastics followed a power law relationship suggesting that plastics could be found at the nanoscale. This was corroborated by a recent study in mussels exposed to the same municipal effluent in this study, which revealed the presence of polystyrene NPs based on fluorescence polarization of an ampiphilic fluorescence probe [3]. The levels of NPs were increased in the digestive gland in mussels caged in the municipal effluent plume compared to the upstream and combined sewer overflow sites (Figure 5A). No NPs were detected in the combined sewers overflow (CSO) site compared to the upstream site. Analysis of the time dependency in NADH formation rate in time revealed a decrease in the slope h in mussels exposed to the downstream site compared to the upstream site (Figure 5B). This indicates a loss of time dependence and

increased in the sD as observed in mussels exposed to NPs. Interestingly, the sD of mussels at the upstream site was circa at 1.5 giving an equivalent percolation environment of 35% space occupancy of the fumarase-MDH-CS reaction in the digestive gland S12 fraction. This corresponds to a drop from 47% to 35% space occupancy between control mussels reared in the laboratory and mussels placed at an upstream site in an urban area. This suggests that the upstream mussels could also be affected by NPs. Nevertheless, mussels placed at the downstream site had significantly higher sD of 1.75 giving an equivalent percolation cluster of 23% occupancy.

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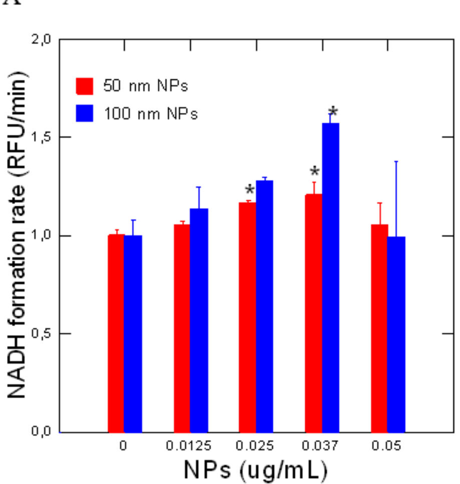

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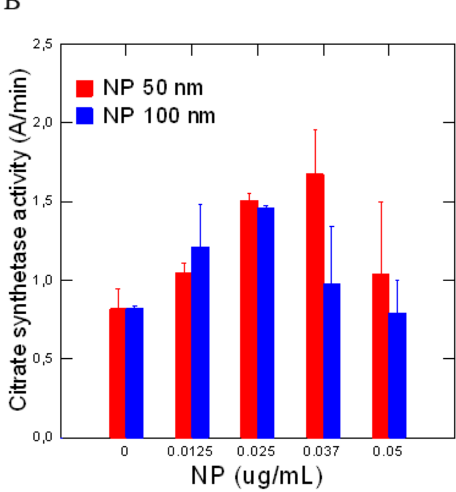

Figure 2. Influence of polystyrene NPs on the MDH and CS activities in vitro.

The enzymes fumarase, malate dehydrogenase and citrate synthase were incubated in the presence of fumarate, NAD^+ and acetylCoA at 1 mM concentration and the formation of NADH in time was determined in the presence of 50 and 100 nm NPs. The rate of formation of NADH from fumarase-MDH (A) and CS activity (B) are shown with the additions of 50 or 100 nm NPs.

This was the strongest increase in sD compared to the mussels exposed to NPs and the in vitro addition of NPs. The changes in the sD could have contributed to increased reaction rates in fumarase-MDH, in part at lease. This was corroborated by the significant correlation (r=0.50) between the fumarase-MDH activity and the sD where the residual activity i.e., the activity not affected by sD, revealed no significant changes between sites. This suggests that the increased in fumarase-MDH activity in mussels at the downstream site was explained by the sD of the cluster. The liaison of α-chemotrypsin to neutral 110 nm polystyrene NPs leads to an increased catalytic rate from 17.8 to 20 sec^{-1} [16], suggesting that increased enzyme activity involves a close interaction between the enzymes and the surface of NPs. The close association of the fumarase-MDH-CS enzyme cluster could favor channelling of the substrates by two mechanisms [17]. The first mechanism of substrate channelling is the formation of tunnels or pores within the complex (having of h=0.5 with an sD=1). The MDH-CS cluster involves also channeling through electrostatic guidance where the negatively charge malate and oxaloacetate are guided by the positive charge at the surface of MDH and CS complex. In this case, an optimal distance between the enzymes in the cluster is required to enhanced activity in order to balance the tunneling effects and electrostatic guidance. Based on the in vitro data, increasing the sD favored the reaction of the complex at sD between 0.9 to 1.2 which corresponds to tunnel or pore like-morphology with an sD=1. Higher levels of sD reduce the activity of enzymes cluster perhaps by weakening electrostatic guidance and increasing random diffusion of the solutes.

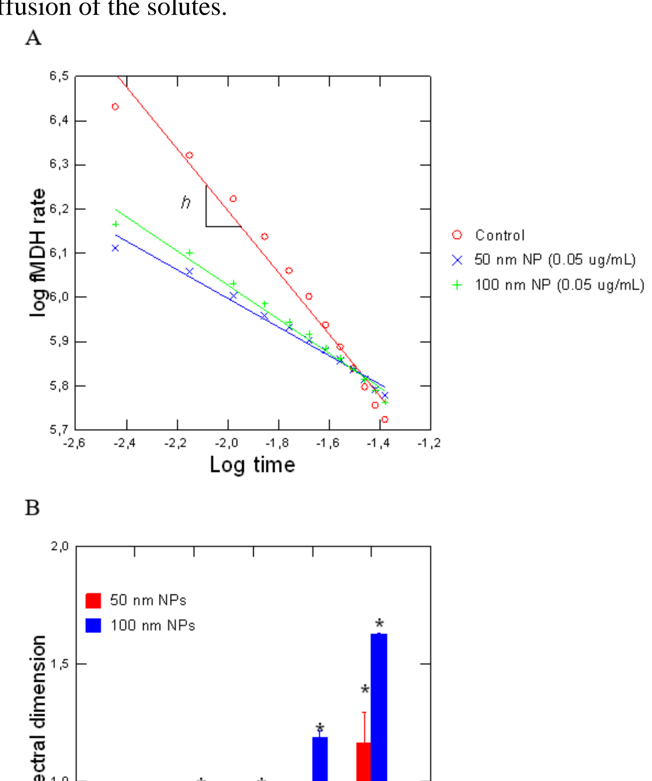

Figure 3. Time-dependent changes in the reaction rates and spectral dimension of the enzymes cluster.

The rate of NADH production was followed in time (A) and the spectral dimension sD was determined (B) in the presence of increasing amounts of 50 and 100 nm diameter NPs. The data represent the mean with the standard deviation. The star symbol indicates significance with respect to controls.

The presence of plastic NPs and perhaps other NPs could disrupt the spatial organization of multi-enzyme clusters such as the fumarase-MDH-CS. From the environmental hazard of NPs perspective, the understanding of the basic properties of NPs such as size/form, surface properties and chemical composition is of importance. Biomarkers of steric interactions of NPs towards enzyme clusters and pathways are needed to better understand the contribution of NPs size/form on the morphological arrangement of

enzymes and protein networks. In the case of polystyrene NPs, these NPs are thought to be relatively stable although they can introduce macroscopic changes in the spatial organisation of proteins in cells.

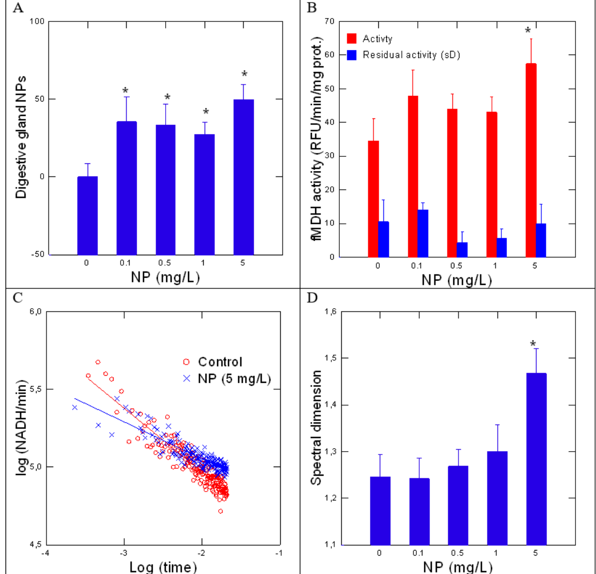

Figure 4. Detection of NPs and MDH activity in mussels exposed to polystyrene NPs.

Mussels were exposed to the NPs for 24 h and the digestive gland was removed for the determination of MDH activity by the addition of "metabolon substrate" mix (fumarate, NAD^+ and acetylCoA). The rate of NADH production over time (A), MDH activity (B) and the spectral dimension (C) were determined.

Based on fractal kinetic analysis, the mere presence of "inert" polystyrene NPs could increase the spectral (fractal) dimension of reactions taking place in these clusters as evidenced by the time dependence in enzyme reaction rates. The increase in the sD suggests that these enzymes are less controlled in time as normally observed in crowded environments surpassing well over reported values for homogenous fractals such as random percolation lattice and tunnels. These effects were observed *in vitro* with commercial preparations of isolated enzymes of fumarase, MDH and CS, which was confirmed *in vivo* in mussels exposed to polystyrene NPs and in mussels exposed to municipal effluents. The decrease in the time dependence in enzyme activity involved in energy metabolism (TCA cycle) just by changing the topological dimensions of the clusters could contribute in maintaining enzyme activity involved in energy producing pathways leading to sustained energy expenses in organisms exposed to NPs. One consequence of the biophysical effects of NPs on enzyme clusters is that they could contribute to depletion of energy reserves under food-limited conditions [18].

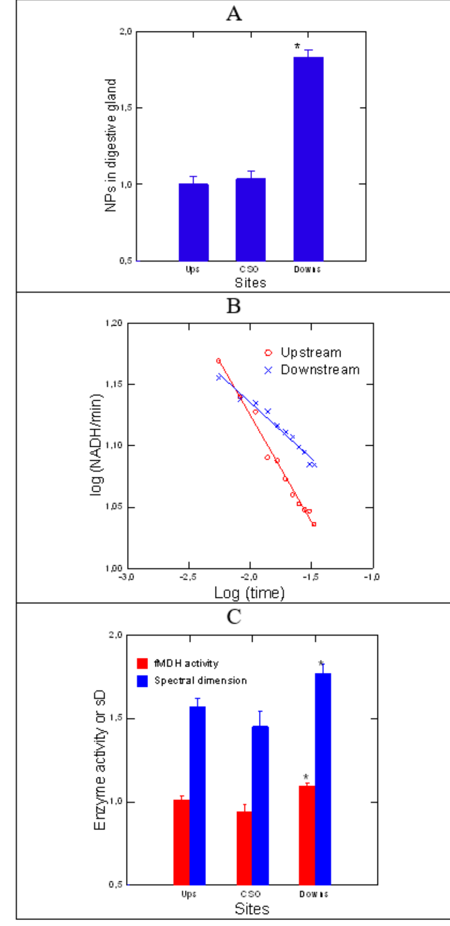

Figure 5. Change in the NADH production rate of the enzyme cluster in mussels exposed to municipal effluents.

Mussels were placed upstream and downstream a municipal effluent dispersion plume and a combined sewer overflow site for 3 months during the summer. The mussels were then collected and the digestive gland isolated for the measurement of NPs in the digestive gland (A) and the determination of the activity of the enzyme cluster. The rate of formation of NADH in time (B), fMDH activity and the spectral dimension (C) were determined. The data represent the mean with the standard error and the star symbol * indicates significance from the upstream (reference) site.

4. CONCLUSIONS

In conclusion, the presence of polystyrene NPs could disrupt the spatial organization of enzyme clusters such as the fumarase-MDH-CS system. The effects of these NPs consisted in the increase in the sD, which suggests a relaxation/dispersion of the enzymes complex. The increase in the sD from 0.58 to >1 suggests a transition from a dust-like organization of the clusters to a connected fractals such as tunnel-like and random percolation structures. These effects were also validated in mussels exposed to either polystyrene NPs in the laboratory and in caged mussels upstream and downstream a municipal effluent dispersion plume. This study confirms that polystyrene NPs could change the space dimensions where enzyme reactions takes place. In the light of the findings in this study, the decrease in the time-dependcy in time (h) caused by polystyrene NPs could maintain high enzyme activity and depleted substrate levels for glucose energy metabolism, which could favor other pathways (i.e., gluconeogenesis). The ecotoxicological consequence of these biophysical effects on the space organization of enzyme clusters will require more research.

5. REFERENCES

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