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ADAPTATIONS OF THREE NEREID POLYCHAETES TO HYPOXIA IN THEIR ENVIRONMENT

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ABSTRACT

A study of the morphological and physiological respiratory adaptations to hypoxia of three nereid polychaete species from Roscoff, France coast axis of the English Channel was conducted. Result of the analysis of their notopodia showed that *Nereis diversicolor* had the greatest specific branchial surface area (SBSA) followed by *Platynereis dumerilii* while *Perinereis cultrifera* had the least. The affinity of their purified haemoglobins to oxygen, interpreted by the values of their P_{50}, also followed the same pattern. The results of this study show that *Nereis diversicolor*, which lives in muddy sediment is better adapted to hypoxia than *Platynereis dumerilii* which lives in tubes under rocks and *Perinereis cultrifera* which lives in sandy sediments under rocks.

KEYWORDS: Adaptations, Polychaetes, Hypoxia, Environment.

INTRODUCTION

Aquatic organisms are often confronted with fluctuations in their physical, chemical and biological environments. In order to survive, organisms adjust, regulate, tolerate and resist these changes (Odiete, 1999). One of the major challenges in the aquatic environment is the depletion of the dissolved oxygen. Some organisms are able to survive the low oxygen by means of specialisation and adaptations, as well as making some extreme changes in their body morphology and physiology (Schneider and Bush-Brown, 2003).

Polychaetes are segmented marine worms that can be found living on the ocean floor or floating in the water column (Rouse and Pleijel, 2001). They are commonly used as environmental monitors because they contact with both the water column and the sediment covering the ocean floor and are sensitive to toxic compounds in both.

The polychaete worms, as benthic fauna are biological parameters that indicate the overall aquatic fertility of the sediments, and the study of the polychaetes may be used as baseline information to evaluate the demersal fish stocks, as they form a major food item in the nutrition of the bottom feeders. In addition, and more importantly they could be used as good biological indicators of marine pollution. According to Mancini *et al*. (1986) and James (1986), 30-50% of the total oxygen intake can be attributed to the sediment oxygen demand (SOD) which is due to various dissolve oxygen consuming reactions that take place in the bottom sediment most of which are by the benthic polychaetes. The Polychaetes, *Capitella capitata, Nereis* sp. and *Polypore* sp. have been observed to colonize hitherto uncolonized anoxic, sulphide clay, bottom substrates, with its characteristic putrefying hydrogen sulphide odour (Ajao & Fagade, 1990).

Nereid polychaetes which commonly live in large numbers in the intertidal zone of estuarine and brackish waters, normally in sediments of medium to high organic contents (Muus, 1967) have been known to be opportunistic, thriving in hypoxic environment and have been used as pollution indicators. There is however paucity of information on the morphological and physiological adaptive features of these polychaetes to hypoxia. This work seeks to investigate the morphological and physiological adaptive features of *Nereis diversicolor*, *Perinereis cultrifera* and *Platynereis dumerilii* in their environment.

MATERIALS AND METHOD

Samples of the three nereid polychaetes, *Nereis diversicolor, Perinereis cutrifera* and *Platynereis dumerilii* were collected from north-west end of France, at the Roscoff coast axis of the English Channel at low tide. *Perinereis cutrifera* and *Platynereis dumerilii* were collected upstream, under rocks while *Nereis diversicolor* was collected downstream, buried in the mud. In the laboratory, representative samples of the three nereid species were preserved at a temperature of -80^0C and studied for morphological adaptive features.

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Morphological Study of the Nereid Parapodia

Representative samples of the nereid worms were fixed in 10% formalin and later rinsed in 3% formalin and in fresh water respectively. The fresh sample of each species was weighed and the weight was recorded before it was gently dried in tissue paper and cut into different fractions of similar widths. Each fraction was viewed under the microscope (Olympus SZX9 Model) and the number of segments recorded. A new scalpel was used to make a transverse section of a segment of each fraction. This was viewed under the microscope and the photograph taken (Plate 1).

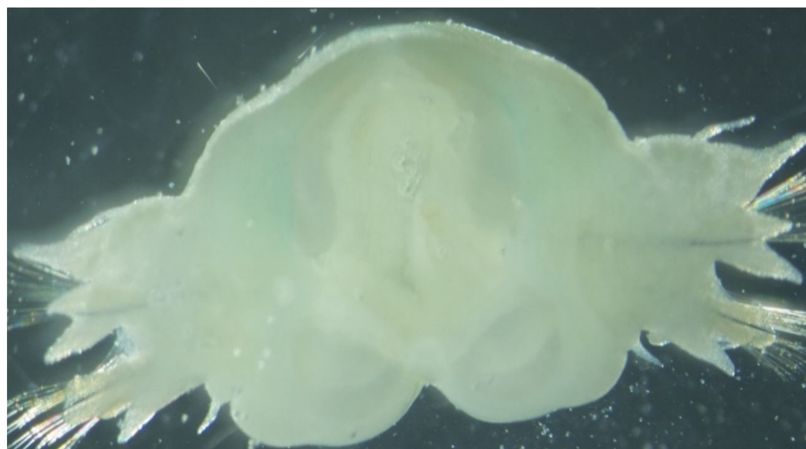

Plate 1: Transverse section of the segment of *Nereis diversicolor* under Olympus SZX9 Microscope Mag. X50

The transverse section of a segment of each fraction was analysed with graphic software (Image J) to obtain the specific surface area of the branchial surface and this was multiplied by the number of segments in that fraction. This analysis was performed for all species and Microsoft Excel was used to calculate the ratio of the surface area of their notopodia (gills) to their entire body surface. A larger notopodia surface area is an indication of better adaptations to trapping of oxygen (Andersen *et al*, 2002).

Physiological Study of the Nereid Polychaetes

The frozen worms were weighed and put in a special Corex glass tube for homogenisation. Equal volume of the Nereid buffer was added and PhenylMethylSolfonyl Floride, a serine protease inhibitor was then added in the ratio of 1ml of buffer: 1µl of PMSF. The mixture was ground in the special tight glass tube always held in ice, and made as homogenous as possible. The ice and the PMSF were to inhibit the action of any enzyme in the sample so as to prevent the degradation of the protein molecules in the sample.

Homogenisation of Frozen Nereid Worms

The homogenate was centrifuged in 15ml Corex tubes at 5000rpm at 4^0C for 5min to pellet the big pieces that are not ground. The supernatant was then centrifuged at 10,000rpm at 4^0C for 30min. The supernatant (H_1) was collected for fractionation with Ammonium sulphate.

A known volume of the homogenate was poured into a small flat-bottomed glass beaker. The beaker was placed in a larger beaker and surrounded with ice cubes. A magnetic bar was dropped into the beaker and it was placed on a magnetic stirrer. Ammonium sulphate was progressively added in the ratio of 0.301g of $(NH_4)_2SO_{4:}$ 1ml of the homogenate within a period of 5minutes. The stirring was done for 30min to allow for proper dissolution of the salt. The solution was centrifuged at 10000rpm, at 4^0C for 10min. The supernatant (red in colour) was pipetted out very carefully, avoiding the pellets and labeled F_1.

The volume of F_1 was measured and it was poured into a beaker. Ammonium sulphate salt was progressively added in the ratio of 0.095g of the salt: 1ml of F_1 within a period of 5minutes. It was then placed in a larger beaker with ice and set on a magnetic stirrer for 30min. After the 30min, it was centrifuged at 10000rpm, at 4^0C for 10min. The supernatant was yellowish green and the pellets very red in colour. This means that all the heamoglobins have been precipitated. Both the supernatant and the pellets (most importantly) were collected. The Pellets was labeled F_2 and the supernatant labeled F_3. H_1, F_1, F_2 and F_3 were stored at the temperature of -40^0C for subsequent analysis with the Fast Pressure Liquid Chromatography.

Purification of Nereid Haemoglobin

Purification of the Nereid haemoglobins was performed by size-exclusion chromatography (SEC) with the Fast Pressure Liquid Chromatography. The precipitated sample which was stored at -40^0C was thawed in ice and

made into solution using 2ml of freshly prepared and ultra-filtered Nereid buffer. Ten aliquots of 205µl each were put into ten different cuvette bottles in readiness for injection into the Autosampler of the FPLC. Before injecting the samples, the system was programmed and the software used for this is called 'Empower'. The proteins in the F_2 were separated by analytical size-exclusion chromatography performed on a high-resolution Superose 6 10/300 Tricorn (GL) column (GE Healthcare), with a fractionation range of 5 to 5000 kDa (Figure 3.3). The flow rate was 0.5 ml/min in saline Nereid buffer. The absorbance of the eluate was monitored at wave lengths of 280nm and 414nm, to measure protein and haem concentrations, respectively. The haem-containing protein was collected separately at the peak of interest and concentrated on micro-centrifugal filter devices Centricon-10 (Millipore).

Oxygen binding at different pH

One of the factors that affect the loading and unloading of oxygen by haemoglobin is the pH (Hourdez and Lallier, 2006). In order to determine the impact of pH on the oxygen binding of the haemoglobin, the purified sample of the nereid species were diluted with nereid buffer of varying pH. The temperature of the diffusion chamber was fixed at 15^0C through the Ministat CC1 temperature regulator. 5µl of the aliquot of the pure sample was dropped and spread on a special slide and introduced into the diffusion chamber under the UV light. The oxygen dissociation curve was generated on the computer. Then 15µl of the pure sample was diluted with equal volume of a Nereid buffer of pH 5.5 and thoroughly mixed using the electrical agitator. 5µl of the aliquot of the diluted sample at pH 5.5 was dropped and spread on a special slide and introduced into the diffusion chamber under the UV light. This was repeated for dilutions at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 respectively. The oxygen dissociation curves generated were further analysed in Microsoft Excel to determine the impact of pH on the P_{50}.

RESULT AND DISCUSSION

Specific Branchial Surface Area

Nereis diversicolor had the greatest specific branchial surface area (SBSA). *Perinereis cultrifera* had the smallest SBSA gill surface. *Platynereis dumerilii* had SBSA less than that of *Nereis diversicolor* but larger than that of *Perinereis cultrifera* (Table 1, Figure 1). With the larger branchial surface area, *Nereis diversicolor* has evolved some morphological adaptive features to hypoxia more than the other two species studied. Unlike *Perinereis cultrifera* which live on sandy surfaces under rocks, or *Platynereis dumerilii* which live in loose tubes under rocks, *Nereis diversicolor* live in hypoxic muddy sediments where oxygen diffusion is restricted, and would therefore need to evolve some adaptations for this condition. Andersen *et al* (2010) had stated that *Nereis diversicolor* was well adapted to its muddy, hypoxic burrows.

Table 1: Analysis of the Branchial Surface of the Three Nereid Polychaetes

Nereid Species	Fresh Weight	Total Surface Area	Total Surface Area without Branchia	Total Branchial Surface Area	Specific Branchial Surface Area
Perinereis cultrifera	0.59g	790.77mm^2	738.56 mm^2	52.21 mm^2	88.48 mm^2/g
Platynereis dumerilii	0.13g	317.49 mm^2	295.53 mm^2	21.96 mm^2	168.92 mm^2/g
Nereis diversicolor	0.45g	815.46 mm^2	729.54 mm^2	85.92 mm^2	191.40 mm^2/g

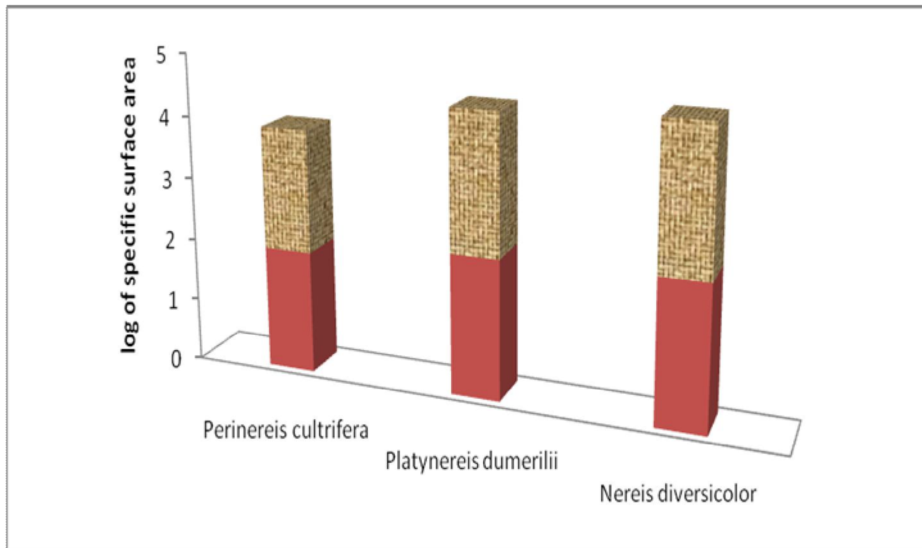

Figure 1: Variation in the Branchial Specific Surface Area of *Perinereis cultrifera, Platynereis dumerilii and Nereis diversicolor*

Purification of the haemoglobin

The chromatogram shows a high concentration of non haem protein in the homogenate as indicated by the red line (absorption at 280nm) with the peak heigl AU. The haem protein on the other hand was not concentrated as indicated by the black line (absorptio n) with peak height of about 0.20AU (Figure 2). This is because at this stage of purification, the heam protein has not been concentrated by either Ammonium Sulphate precipitation or Fast Pressure Lipid Chromatography.

In Figure 3, the heam protein of the Nereid polychaetes has been concentrated through the process of precipitation by Ammonium Sulphate salt as could be seen in the peak height (0.42AU) of the black line (absorption at 414nm). The flat black line (absorption at 414nm) at zero AU in Figure 4 indicates the near total absence of the haem protein in the aliquot. This fractionate (F_4) was the supernatant collected after the proteins have been precipitated and collected as in F_3 (Figure 3). Fractionate 4 was however discarded and no collection at the peak of interest was conducted as it contains little or no haemoglobin. The observed peak which was for non haem proteins, formed after a relatively long period (40 minutes) unlike that of the haemoglobin which forms not later than 20 minutes (see Figure 3 and Figure 4 respectively). This is because the non haem proteins are smaller than the haemoglobins and have smaller molecular weight, and as a result, elute later than the haemoglobins in the column.

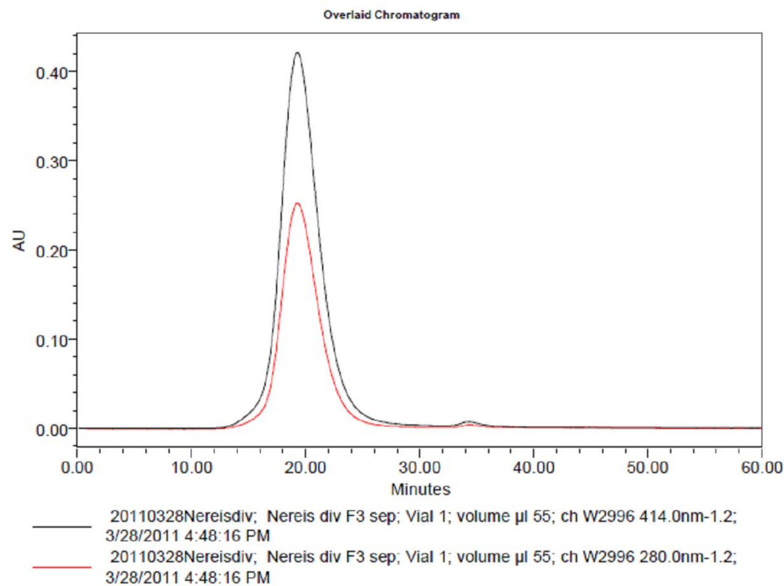

Figure 2: Chromatogram of *Nereis diversicolor* Homogenate for the Purification of the hemoglobin

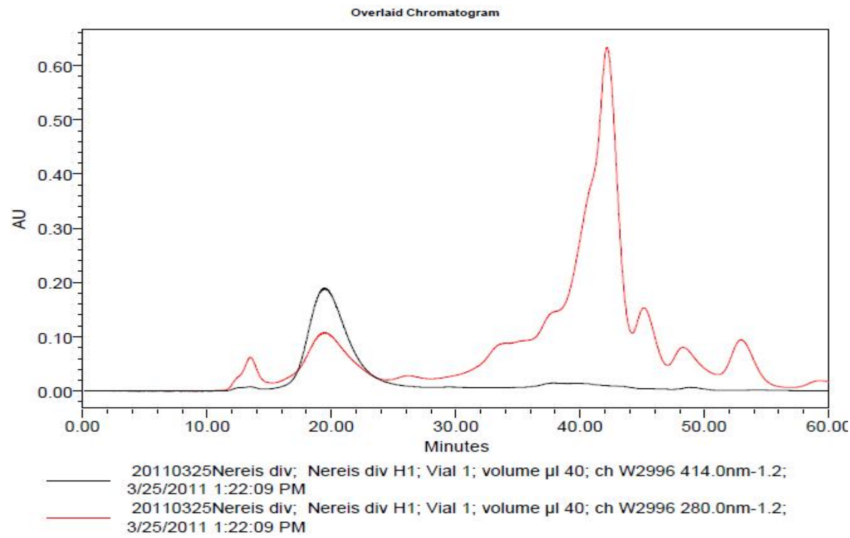

Figure 3: Chromatogram of *Nereis diversicolor* Fractionate (F_3) for the Purification of the hemoglobin

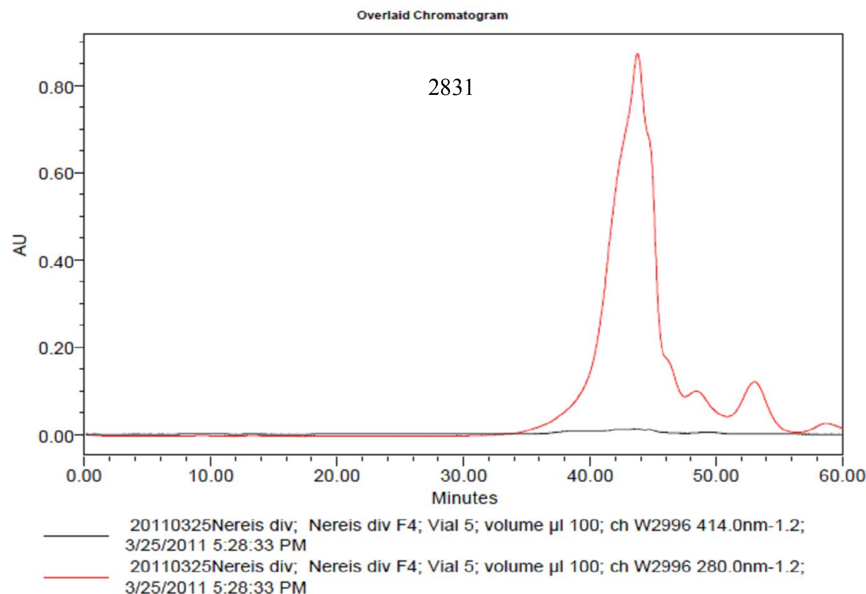

Figure 4: Chromatogram of *Nereis diversicolor* Fractionate (F4) for the Purification of the hemoglobin

Oxygen Binding Properties of the Nereid Species at Different pH

Table 2 and Figure 5 show a decrease in the P_{50} values with increase in the pH in the haemoglobin of the three nereid polychaetes studied. This means that an increase in the pH (low acidity) leads to increase in affinity of the haemoglobin to oxygen, and conversely, a decrease in the pH (high acidity) leads to decrease in affinity. High concentration of the hydrogen ion (H^+) compels the haemoglobin to release the oxygen molecules that bind to it to the fluid that surrounds it and this leads to decreased affinity. The acidic tissues require oxygen and therefore, the haemoglobin is compelled to release oxygen and thereby reducing the oxygen that is stuck to it. This is known as the Bohr Effect. At acidic medium (low pH) therefore, there is low affinity (and high P_{50}).

In *Perinereis cultrifera*, the least affinity was observed at pH of 6.4 and it continued to increase with an increase in pH but in *Platynereis dumerilii* and *Nereis diversicolor*, the least affinity was observed at pH of about 6.5 and continued to increase with increase in pH (Table 2). The cause of this slight difference in the trend of their affinity with pH is not well understood, but could be attributed to the differences in their ecological niches. While

Perinereis cultrifera inhabits well ventilated, sandy surfaces under rocks, *Platynereis dumerilii* and *Nereis diversicolor* inhabit not well ventilated silky tubes, and hypoxic muddy sands, respectively.

Table 2: Haemoglobin Oxygen Affinity of the Three Nereid Species at varying pH and at Temperature of 15°C

Perinereis cultrifera			*Platynereis dumerilii*			*Nereis diversicolor*		
pH	**P_{50}**	**log P_{50}**	**pH**	**P_{50}**	**log P_{50}**	**pH**	**P_{50}**	**log P_{50}**
6.4	46.2	1.65	6.4	17.2	1.236	6.4	8.2	0.914
6.7	38.8	1.589	6.6	20.6	1.314	6.5	11.8	1.072
7	27.3	1.436	6.9	17.6	1.246	6.9	8	0.903
7.3	11.6	1.064	7.4	10.4	1.017	7.4	5.9	0.771
7.7	8.8	0.944	7.6	6.5	0.813	7.7	5	0.699
8.1	7.2	0.857	8	3.9	0.591	7.8	5	0.699
8.2	5.1	0.708	8.3	3.2	0.505	8.2	2.7	0.431

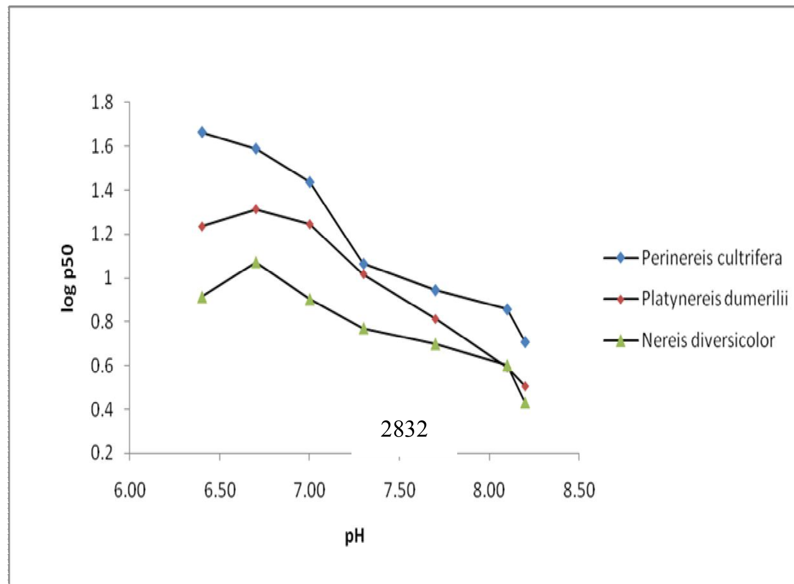

Figure 5: Comparison of the P_{50} values in the haemoglobins of the three Nereid species at different pH and at 15°C

At the same pH, the three nereid polychaetes haemoglobin showed differences in their oxygen affinities. *Perinereis cultrifera* showed the least affinity and followed by *Platynereis dumerilii*. The highest affinity was exhibited by *Nereis diversicolor*. Their affinities to oxygen is a respiratory adaptation to the ambient oxygen tension. *Nereis diversicolor* lives in burrows under mud where hypoxia prevails. It would have therefore evolved some adaptations to this low oxygen, hence the increased affinity. *Perinereis cultrifera* lives on the surface of well ventilated sandy sediment, sheltered by the rocks. This species, as result of the type of sediment and positioning in its habitat, experiences no pressures of low oxygen. *Platynereis dumerilii* is in between of the two. It lives in silky tubes which are not as ventilated as the sandy sediment surface in the *Perinereis cultrifera* and not as hypoxic as in the muddy burrows of *Nereis diversicolor*.

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