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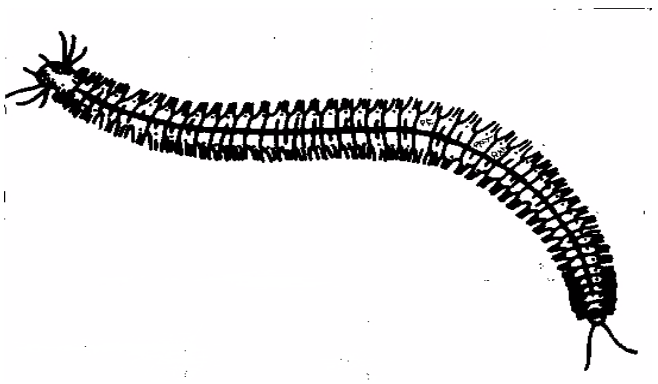

The influences of sewage pollution on *Nereis diversicolor*

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Table of Contents

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1. Abstract

Stable isotope analyses have been used to determine the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope signatures of: the polychaete worm *Nereis diversicolor*, sediments and phytoplankton on different sites in South East England. *Nereis* are found in shallow and brackish waters in Europe and North America, and serve as good biological models used in different fields of research for many experimental studies. They are omnivores; they can either be deposit feeders when tide is in or out, using organic material in sediments as food, or suspension feeders when the tide is in, secreting a mucous net to trap phytoplankton. Most recent studies by Hughes & Paramor 2004, have showed that bioturbation and herbivory by *Nereis* cause loss of pioneer zone plants, hence increase sediment instability and intensify the erosion of saltmarsh creeks. For the past 50 years approximately it is believed that infauna have changed from bivalve to polychaete dominance, due to increase in eutrophication, of sewage deposit of particulate organic matter and nutrient enrichment by microphytobenthos.

This study concentrates on the influences of sewage pollution because *Nereis* serves as a good bio-monitor of pollution in different sites as sewage-derived carbon and nitrogen remains in the polychaete worm tissue as a result of enrichment in animal's $\delta^{13}C$ and $\delta^{15}N$ versus diet.

This study will show that *Nereis* from sewage affected sites will have nitrogen isotopic signatures different to those from clean sites, which reflect their greater dependence on deposit feeding, rather than filter feeding typical in clean sites.

Key-words: stable isotope analysis, *Nereis diversicolor*, sewage pollution, bioturbation, deposit feeder

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2. Introduction

Saltmarshes are intertidal regions on sandy or muddy sheltered costal areas and in estuaries, where freshwater and seawater meets, supporting characteristic plant and animals communities containing rare species of plants and invertebrates (Doody 2001). There are communities of emergent herbs, grasses, or low shrubs rooted in soils alternately inundated and drained by tidal action. They are halophytes, meaning they grow in soils with high salt concentration. They occur mainly at the higher tidal levels in areas of protected water and most often in association with estuaries.

The definition of coastal saltmarsh stresses that the flooding waters are saline, not necessarily seawater but more saline than freshwater (Long & Mason, 1983. Adam, 1990). The source of sediment deposited on saltmarshes by the tides is erosion of terrestrial rocks. In addition to saltmarsh sediments often there is calcium carbonate fragments from molluscan shells, either from animals living in the marsh or from offshore mudflats, where in some cases the carbonate may constitute a high proportion of the sediment. Coastal saltmarsh occupies the interface between sea and land, and there are numerous interactions between the animals of both areas and the marsh plants and their environment, which reflects its biota, both flora and fauna, has both marine and terrestrial components. The primary producers of the marsh are the emergent marsh plants and the various microalgae that live on the surface of the plants substrate. The major terrestrial animal components of marshes are insects, and the marshes are invaded at high tide by marine and estuarine animals. Few herbivores consume marsh vegetation, possibly because marsh plants have high salt content. Marsh plants are beneficial to the animals in a number of ways including providing cover that reduces predation, stabilizing the substrate and providing a surface for attachment, and also reduces wave and current action. In relation to animals, these ones have effects on the plants including aerating the substrate by burrowing, grazing off epiphytic algae, providing nutrients through faecal deposition, and in the case of mussels and oysters, stabilize the substrate. So, saltmarsh is best regarded as a highly modified terrestrial ecosystem (Adam, 1990).

The abundant invertebrate community in saltmarshes is a vital source for the internationally important wading birds found on the Essex and Kent coast. However, saltmarshes within and around the UK have been eroding rapidly for the past 50 years

(Paramor & Hughes, 2004) as loss is estimated to be at a continuing rate of about 40 ha $year^{-1}$. 60 % of which are lost from Essex Estuaries European Marine Site/Ramsar Site (Paramor & Hughes, 2004). This is arguably the most important habitat conservation problem in the UK, not only because of the importance of the marshes and the high rate of loss, but because of the lack of understanding of the actual causes and appropriate solutions (Paramor & Hughes, 2004). The potential sources of energy in the invertebrate food webs of the estuarine ecosystems are from the saltmarsh flora itself, or from phytoplankton deposition, opportunistic algae in the enriched estuarine water, or from terrestrial sources such as sewage. Phytoplankton deposits on saltmarshes have been found to be very low and would not be able to make up for the lost saltmarsh area. The erosion of marshes may have increased the overall input of detritus into the system, or terrestrial sources may be acting as buffers, continuing to feed the systems independently of the inputs from the saltmarsh itself. Saltmarshes are areas of high primary productivity and are very important habitats for nature conservation and also coastal defence, as they offer some protection to sea walls from wave action (Hughes & Paramor 2004). These areas are of international conservation importance, largely because of their significance in estuaries used by resident, migrating and overwintering birds (Hughes 2004), and more than 80% are covered by one or more national or international conservation designations.

This study concentrates on different saltmarshes areas of South Eastern England and a common ragworm *Nereis diversicolor* a well-known polychaete worm, serves as good biological models because they are able to tolerate great variations of temperature (Ivleva, 1970; Wolff, 1973), salinity (Wolff, 1973; Neuhoff, 1979) and survive hypoxic conditions (Wells & Dales, 1951; Kristensen, 1983). *Nereis* are used in different fields of research for many experimental studies, as a consequence, this species is able to settle in naturally fluctuant environments such as the upper waters of estuaries. The range of this species is restricted to the shallow marine and brackish waters in the North Temperate Zone from both Europe and North America. *Nereis* are one of the few polychaete species to present a commercial interest; it is dug from intertidal mudflats and sold as bait in recreational fishing and as food in aquaculture (Scaps 2002).

The common ragworm is an infaunal species that inhabits sandy muds but also gravels, clays and even turf (Clay, 1967) where it builds U or Y-shaped burrows in soft sediments (**Fig 1**) at about 5 to 10 cm deep at densities of up to 4000 m^{-2} (Davey 1994). Burrows depth increases with body size and seasonal variation in burrow depth is correlated with sea temperature (Esselink & Zwarts, 1989). Adult *Nerei*s are territorial and defend their burrow against intruders (Lambert & Retiere, 1987). *Nereis* are gonochoristics species, having sex chromosomally determined as either female or male. As a result, sexual reproduction is present where colour differences appear between the mature individuals. Immature individuals assume a reddish brown colour, whereas the mature males have a bright grass-green colour and females have a much darker green colour. The white mass of sperm that packs the coelom of the male gives it its lighter green colour (Dales, 1950; Scaps, 2002).

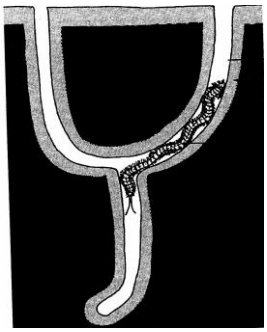


Figure 1: *Nereis* using U or Y-shaped burrows in soft sediments, that are well adapted to verticalize water-sediment interfaces into black anoxic sediments by active ventilation of its burrow (From Scaps 2002).

Nereis has a "benthic-pelagic" life cycle with a short pelagic larval period, eggs are large (egg diameter >200 μm) and are laid by female inside the burrow before male ejects his sperm into free water in front of the gallery. Fertilised eggs remain in the burrow, to be brooded by the female, and during incubation, larvae and post-larvae remain under the body of the female for at least 10 days (Bartles-Hardege & Zeeck, 1990; Scaps, 2002;) and in most populations, maturity takes 1 and 2 years before spawning.

Nereis are eaten by overwintering and migrating birds (waders) by eating ragworms, epibenthic predators, such as larger crabs (*Carcinus maenas*), shrimps and small fish (gobies).

*Nerei*s are omnivores, feeding on algae, detritus, grass seeds and sediment, but is also a predator feeding on other invertebrates. They may behave as a deposit feeder (Reise, 1979) when tide is in or out using two tactics. The first tactic consists in collecting food near its burrow opening (**Fig. 1A**), where they use organic material in

the sediments as food, catching it with the jaws and ingesting it immediately. The second tactic consists of capturing food with mucous secretions (**Fig. 1B**), then when the worm retreats into its burrow, bringing back the mucous secretion that forms into a pellet, then, the worm progressively ingests the pellet within its burrow. However there is another way, in which *Nereis* behaves as a secondary filter feeder (suspension-feeder), eating when the tide is in, where they secrete a mucous net that traps particles such as phytoplankton, carried into the burrow by water circulation. (Harley, 1950; Goerke, 1966; Riisgard, 1994; in Scaps 2002).

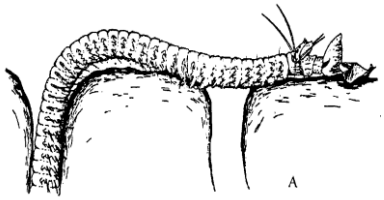
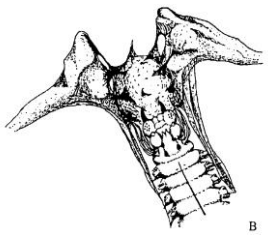


Figure 1A: *N. diversicolor* using 1st tactic without mucous secretion, immediately ingestion of food particles (Scaps 2002).

Figure 1B: *N. diversicolor* using 2nd tactic (suspension-feeder) with mucous secretions collecting food particles brought back in a pellet form to the opening of the burrow (Scaps 2002).



There is some evidence that *Nereis* has become more abundant and widespread in the past few decades (Hughes, 1999). The results of Paramor and Hughes (2004) confirm that bioturbation and herbivory by the infaunal polychaete worm *N. diversicolor*, cause loss of the pioneer zone vegetation, increase sediment instability and intensify the erosion of saltmarsh creeks, so they have identified a major cause of an important economic and habitat conservation problem.

In South East England *Nereis* feeds an important part of detritus based food webs, they may depend on the primary production from (1) saltmarshes (productivity 1400-3500g.m^{-2}.y^{-1}), (2) terrestrial sources (mostly from sewage effluents), (3) the loss of saltmarsh biomass (i.e.40 ha.y^{-1}), (4) microphytobenthos (productivity 29-234g.m^{-2}.y^{-1}) and (5) phytoplankton.

As a result, for the past 50 years approximately, it is believed that infauna have changed from bivalve to polychaete dominance due to increase in eutrophication, of sewage deposit of particulate organic matter and nutrient enrichment by microphytobenthos. The Jennigs et al 2001 study (Impacts of trawling

disturbances on the trophic structure of benthic invertebrate communities at Silver Pit and Hills of the central North Sea), shows that there is a decrease in biomass and an increase in polychaete due to aggregation of more silt sediments and less sand sediments giving then, more favourable conditions for polychaete. The recent saltmarsh erosion may have increased the overall input of detritus into the system, or sewage of the terrestrial sources, such as fertilizers, will encourage the increase of productivity and may be acting as buffers, continuing to feed the system independently of the inputs from saltmarsh, possibly causing the sites to become eutrophic. Sewage effluent discharged into costal environments comprises a significant anthropogenic source of organic matter to the marine load (Rogers, 2003).

This study used stable isotopes in order to identify the variations in carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) signatures of the potential food sources consumed by *Nereis*. An isotope is a form of a chemical element having the same atomic number (Protons) an identical chemical property as another, but differing in atomic mass as a result of a different numbers of neutrons in the atomic nucleus.

$^{12}C\ ^{6}$ ➔ $^{13}C\ ^{6}$ $^{14}N\ ^{7}$ ➔ $^{15}N\ ^{7}$ Atomic mass X Atomic number

There has been an increase in the development of techniques for the use of natural abundance isotopes in ecological research. Indeed isotope analysis is becoming a standard tool for scientists, understanding the diet of an individual and following through its cycle, either aquatic or terrestrial one can understand the ecosystems (use it to study elements or materials cycles in the environment). Natural abundance isotopes can be use to find models and mechanisms within a single individual, plant or animal as well as to trace food webs, and follow whole ecosystem cycling in both terrestrial and marine ecosystems.

The stable isotope theory is that ratios are measured by means of an isotope ratio mass spectrometer that quantifies the ratio of the heavy and light isotopes in a sample and compared to a standard. One can measure the absolute abundance of the isotopes within a sample, but these differences are fairly small and are subjected to problems, such as sample heterogeneity, everyday fluctuations within the mass spectrometer and sample preparation (Hayes, 1982). Consequently, the isotope ratio of a sample (R_{sa}) needs to be compared to a standard (R_{std}), so that fluctuations

would be reflected equally in both sample and standard. The 'R' is the expressed ratio of the heavy to light isotope, and the differences of ratios are calculated in 'delta' (δ) notation with units of per mil (‰).

$$\delta\ (‰) = R_{sa} / R_{std} - 1) \times 1000$$

Samples that contain less of the heavy isotope are referred to as 'depleted' and are 'lighter' than other samples, and those samples that contain more of the heavy isotope are referred to as 'enriched' and are 'heavier' than other samples. (Lajtha & Michener, 1994).

Ecologists are mostly concerned with stable isotopes of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), sulphur ($^{34}S/^{32}S$), oxygen ($^{18}O/^{16}O$) and hydrogen ($^{2}H/^{1}H$), but this study will only consider the stable isotope signatures of carbon and nitrogen. All the reported isotope values in the literature are referenced to primary standards, consequently, for carbon is a marine limestone fossil, Pee Dee Belemnite (PDB) (Craig 1953) and for nitrogen is the atmospheric air (Mariotti, 1983). In food webs analyse the interpretation of these ratios relies on the assumption that the isotopic composition of the animals reflects that of its diet (Gannes *et al*. 1997.).

There are many ways that isotope samples can be analysed and prepared. The purpose is to convert a sample quantitatively to a suitable purified gas (H_2, CO_2 and N_2), which the mass spectrometer can then analyse. Therefore, using a mass spectrometer it is possible to determine $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios and determine the source of productivity in food webs (Haines & Montague, 1979. Peterson & Fry, 1987. Eggers & Jones 2000. Riera & Hubas 2003). The isotopic signatures are modified as organic matter is transferred from one trophic level to the next, and marine studies have consistently shown an increase in ^{13}C ratio of 0.5-1‰ and in ^{15}N ratio of 3.2‰ (Peterson & Fry, 1987., Riera, 1998., Eggers & Jones 2000). Hence if an animal is feeding on sediment or phytoplankton its signature should be similar to that of the sediment or phytoplankton but displaced by 0.5-2‰ for carbon and 3-5‰ for nitrogen (**Fig. 2**). Sewage typically gives a heavier nitrogen signature than terrestrial plants or aquatic sources, and this is because of the high levels of microbial conditioning that it has undergone (Kikuchi & Wada, 1996. Riera et al., 1999. Waldron et al., 2001).

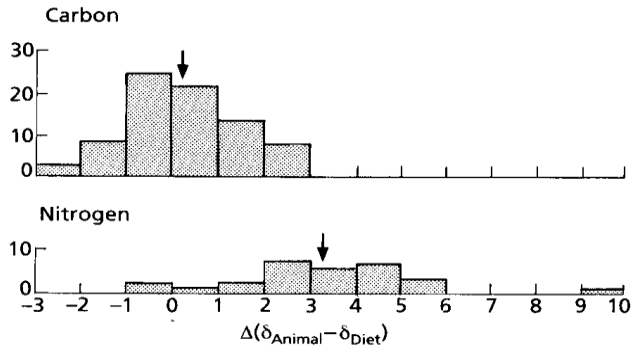


Figure 2: The relationship between organisms and diet for carbon and nitrogen isotopes. (From Peterson & Fry, 1987).

It is possible to see the relationship between organisms and diet in food webs and whether or not there is significant assimilation of nitrogen and carbon from sewage by *Nereis* or indeed from other sources of primary production. Through this process it will be possible to differentiate the diet of *Nereis* from terrestrial sources such as sewage or even between sediments and phytoplankton. If this is the case that *Nereis* are gaining energy from sewage on sediments then increased sewage may well result in high densities of *Nereis* leading to consequences in the saltmarsh erosion.

The research used a variety of sites in the Essex Estuaries European marine Site. The different sites allow comparisons of the effect of sewage inputs on the isotopic signatures of Nereis and their potential food sources. Some of the samples sites were within the vicinity of sewage treatment works (STW) and /or their outfalls. Where other samples sites were at a considerable distance (dozens of km) from STW and/or their outfalls. Samples of Nereis and their potential food sources were collected from these sites.

Those sampled sites which are a considerable distance away from STW and /or their outfalls are considered to be unaffected or indirectly affected by sewage are: Brancaster (Br) (**Fig. 3**), Felixstowe Ferry (F) (**Fig. 4**).

Those sites sampled which were in the vicinity of STW and /or their outfalls are considered to be directly affected by sewage are: Ipswich (I) (**Fig. 5**), Martlesham Creek (MC) (**Fig. 6**), Tollesbury (T) (**Fig. 7**), Grays (G) (**Fig. 8**), Wells Next-the –Sea (W) (**Fig. 9**), Gold Hanger (GH) (**Fig. 10**) and Osea (O) (**Fig. 11**).

Figure 3: Brancaster (Grid Reference: TF 780443) presumably clean site.

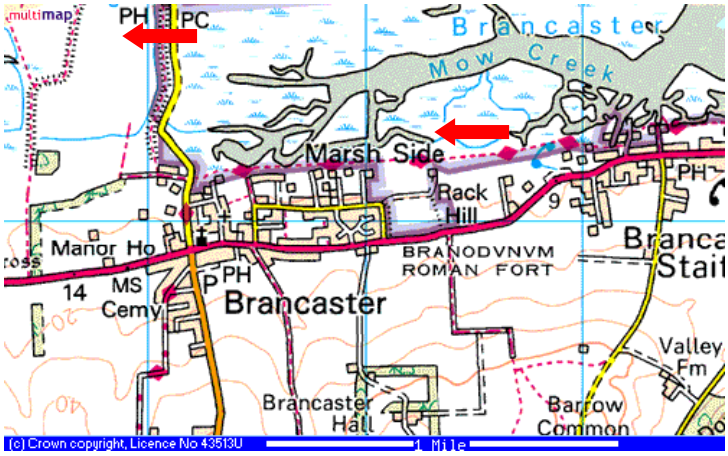


Figure 4:Felixstowe Ferry (Grid Reference: TM324370) presumably clean site.

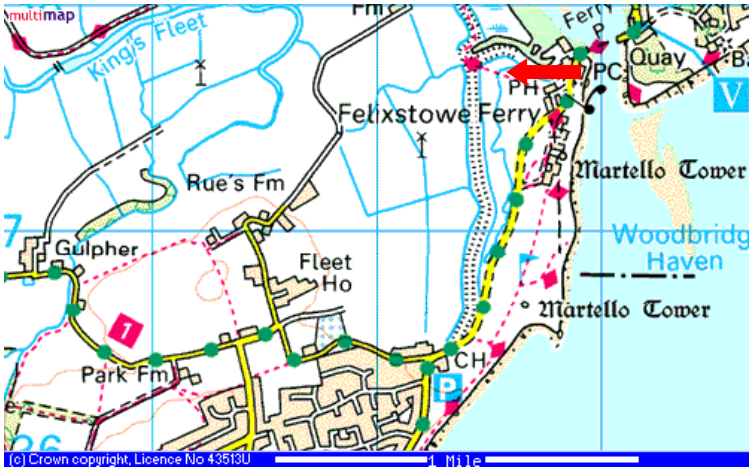


Figure 5: Ipswich (Grid Reference: TM 170410 affected and near the STW

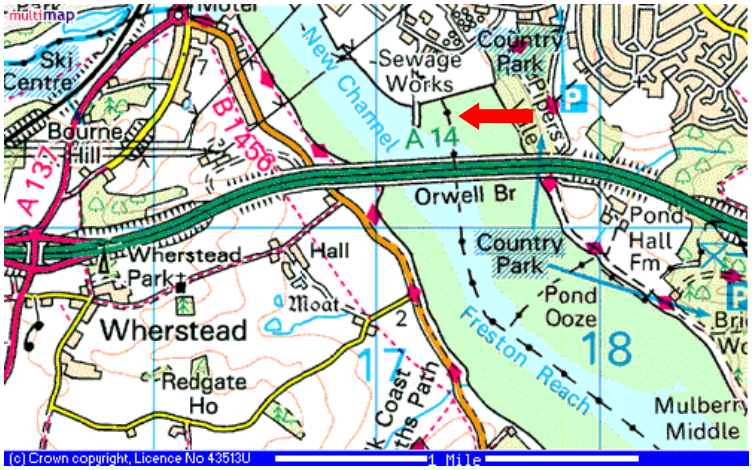


Figure 6: Martlesham Creek (Grid Reference: TM258447) affected by STW

Figure 7: Tollesbury (Grid Reference: TL 964112) clean site but slightly affected by sewage

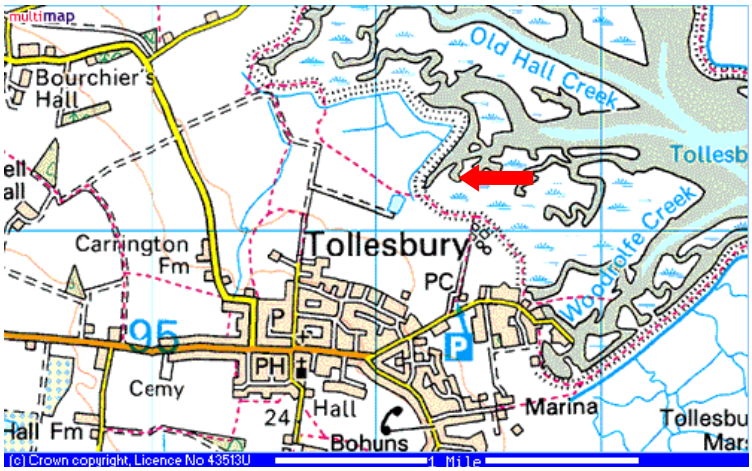


Figure 8: Grays (Grid Reference: TQ608775) affected by STW

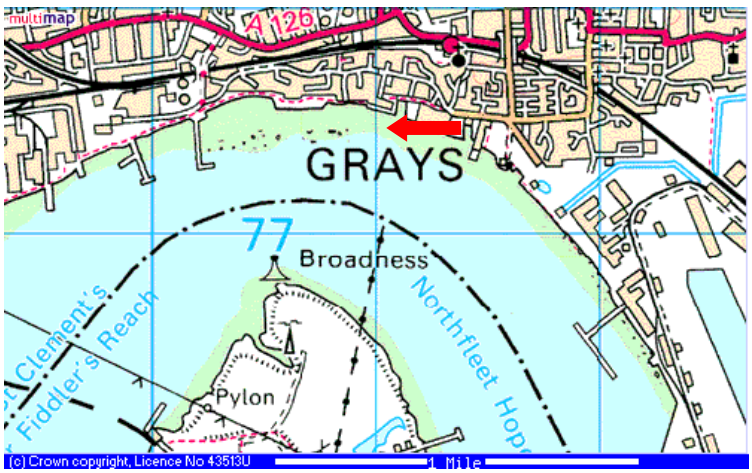


Figure 9: Wells-Next-the-Sea (Grid Reference: TF 920437) slightly affected by sewage

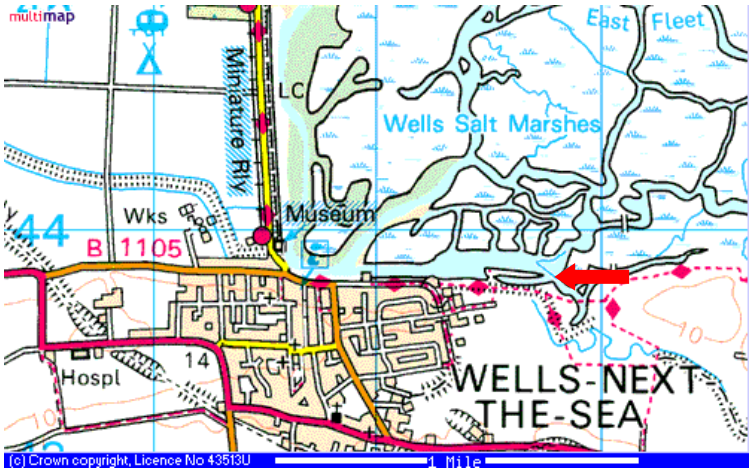


Figure 10: Gold Hanger (Grid Reference: TL 896076) affected by STW

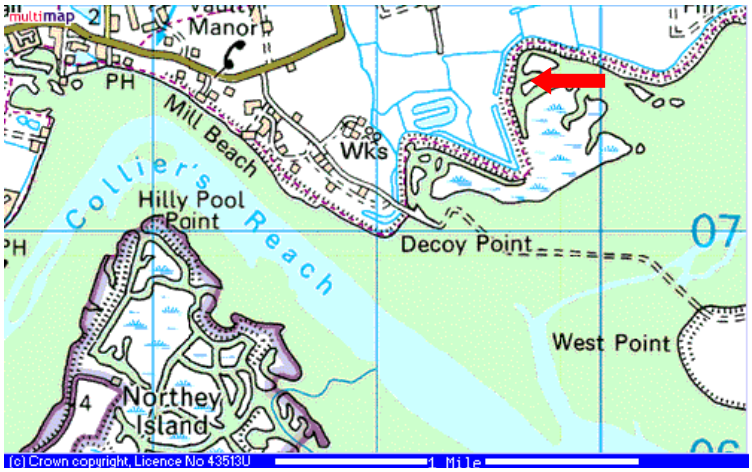
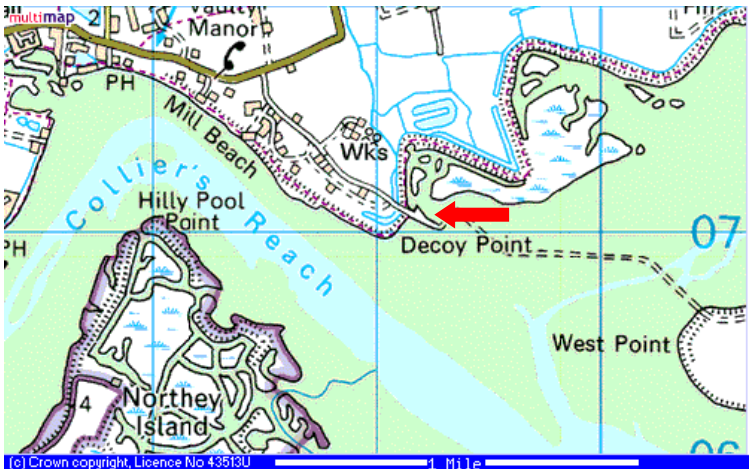


Figure 11: Osea (Grid Reference: TL 892070) affected by STW



The proposed hypothesis for this study is:

Hypothesis: In sewage affected sites will have nitrogen isotopic signature different to those from clean sites, which reflects their greater dependence on deposit feeding rather than filter feeding typical in clean sites.

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3. Methods

Five samples of *N. diversicolor*, sediments and phytoplankton if available, were collected from various different sites, between October 2004 and January 2005. These various different study sites were:

Brancaster, (B) (**Fig. 3**);Brancaster Realigment Site, (Brs); Felixtowe, (F) (**Fig. 4**); Goldhanger, (GH) (**Fig. 10**); Grays, (G) (**Fig. 8**); Ipswich, (Ips) (**Fig. 5**); Martlesham Creek, (MC) (**Fig. 6**); Osea, (O) (**Fig. 11**); Tollesbury Dry, (TD) & Wet, (TW) (**Fig. 7**); Wells High, (WH) & Low, (WL) (**Fig. 9**). The collection of samples at Tollesbury (TD & TW) and Wells (WH & WL) was based on two aspects; Dry (TD) and High (WH) that equals to less low tide and more deposit feeding is possible, compared to Wet (TW) and Low (WL) that equals to more low tide and probably less deposit feeding and more filter feeding.

All samples were placed into a labelled collection tubes. The surface sediment (including particulate detritus and the microphytobenthos available to worms) was collected by scraping the top surface layer (1-3 mm depth) with a knife, while the worms were picked one by one and then inserted into a labelled collection tube. The phytoplankton was also collected when possible, as high tide was needed (while low tide for worms and sediment). All samples were taken to the laboratory at Queen Mary, University of London; the collected worms were placed in a bowl with clean seawater for approximately two hours (plus the time that took them to arrive to the lab approximately 2-4 hours) and this would ensure that the worms' gut were completely emptied, therefore the isotopic signature given by the worm will be indicative of the carbon and nitrogen that has been assimilated into its body and not skewed by the contents. All samples were temporarily frozen until further laboratory analysis. The samples were analysed after defrosting the samples at room temperature (approx. 20°C), all organic samples were dried into a 60°C oven for at least 24 hours.

The oven was not set at higher temperature because otherwise would burn off the organic matter from the samples, hence affecting their isotope signatures. Once dry all the samples were individually homogenised with a pestle and mortar. Samples can then be stored indefinitely in closed containers. In order to avoid contamination between different samples the instruments were rinsed in pure methanol (CH_3OH) to remove any remaining substrate and were allowed to dry before being reused. (For future reference, it is best to use more than 1 mortar and pestle because it takes time

for it to dry, like so less time is lost.) Once homogenised 50 – 100 μg of substrate was weighted out into glass scintillation vials. Sediment and phytoplankton samples were divided into two parts, one to be acidified and the other one not to be acidified. This is because acidification would affect the nitrogen signatures in the isotope ratio mass spectrometer (IRMS) analysis. The presence of $CaCO_3$ in a sample would change the isotopic signature for carbon therefore it must be removed by treating the samples with hydrochloric acid (HCl). 50 –100 μg of sample was placed into glass scintillation vials, to which 1.75ml of 1M HCl was added with a small (2ml) syringe. These samples were then left to stand for 24 hours and then further 0.25ml of 12M HCl was added to ensure that all the $CaCO_3$ had been removed. Then it was once again left to stand for further 24 hours and then the samples were placed in the oven at 60°C for further 24 hours, allowing CO2 to escape and water to evaporate. Samples were then left in the lab with lids on (semi closed at all times, to avoid anything that could fall into the samples vials that could cause contamination) for 24 hours to allow the hydroscopic salts to reach equilibrium (Hedges& Stern, 1984). Therefore the non-acidified samples would give the nitrogen isotopic signatures while the acidified samples would give the carbon isotopic signatures. The reason that part of the samples need to be acidified is because that those samples may contain inorganic carbon, primarily in the form of $CaCO_3$ (Calcium Carbonate).

Once the procedure for $CaCO_3$ removal had been completed the samples were weighed into 30mg atomically pure tin capsules. For all the sediment and phytoplankton samples (acidified and non-acidified) 12mg was weighed and for the *Nereis* samples, 0.8mg was weighed. (To ensure accurate weights a milligram and a microgram weighting scale was used.) Tweezers were used to fold the tin capsules (by carefully folding it) each sample into pellets. After each tin capsule was made into a pellet the instruments were cleaned with methanol in order to avoid contamination of subsequent samples.

A set of urea standards is required every time a set of samples is run through the isotope ratio mass spectrometer (IRMS) analysis. In order to calibrate the mass spectrometer for analysis a urea calibration series was prepared, a 0.1M urea $CO(NH_2)_2$ solution was made up by using 0.6g of urea crystals in 100ml of distilled water (See Box 1& 2) and placed within ultra-clean tin capsules by using a 250 μm pipette it was added 0, 5, 10, 40, 70 and 100 μl of the 0.1M urea standard to clean tin

caps. After urea samples were measured out the samples were then placed within a drying oven at 50 °C. After the samples were placed in the IRMS, and when the data had been obtained from mass spectrometer, it was needed to plot a graph for each nitrogen and carbon with y = area under curve (value of the area from IRMS) and x = mass of the element, so that a good R^2 linearity trend could be obtained and consequently the trend line formula could be used to give a more precise and accurate value for the samples (For nitrogen see example 1).

Note: Instead of 5 samples to be analysed in the mass spectrometer machine, there were only possible to do 3 samples, due to the lack of availability of the mass spectrometer machine, that was booked to other research personal, therefore not enough time to do all the samples.

Box 1 – making up a 0.1M urea solution

Atomic mass = AM

AM Carbon = 12.0110
AM Oxygen = 15.9994
AM Nitrogen = 14.0067
AM Hydrogen = 1.0079

AM Urea $CO(NH_2)_2$
= 12.011 + 15.9994 + ((14.0067 +(1.0079 x 2) x 2)
= 60.0554

IM urea = 60.6g in IL distilled water
0.1M urea = 6.06g in IL distilled water or
0.1M urea = 0.6g in 100ml distilled water

Box 2 – calculating mass of C and N in urea

The proportion of carbon in urea
= 12.0110/60.0554
= 0.1999
The proportion of nitrogen in urea
= (14.0067 x 2) /60.0554
= 0.4665

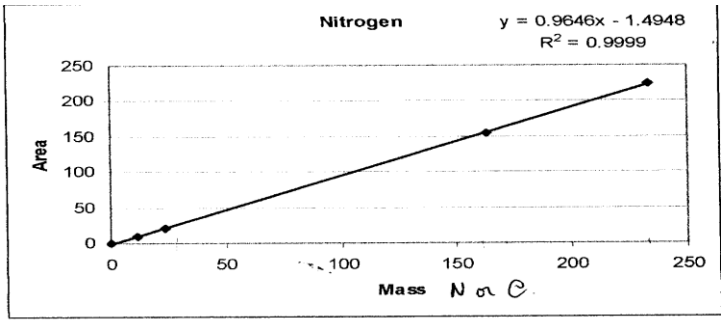
In a 5ul sample of 0.1M urea the total carbon and nitrogen is calculated as follows:
0.1M urea contains 0.6g urea in 100ml or
0.1M urea contains 0.6g urea in 100,000ul
Thus, in a 5ul sample the total urea will be
5/100,000ul * 0.6g
= 3 x 10^{-5}g or 30ug urea

Of this
Carbon = 30ug x 0.1999 = 6ug
Nitrogen = 30ug x 0.4665 = 14ug

3. The full data set for the 0.1M (i.e. 0.6g in 100ml) urea standard is given in the table below (see Box 2 for calculations):

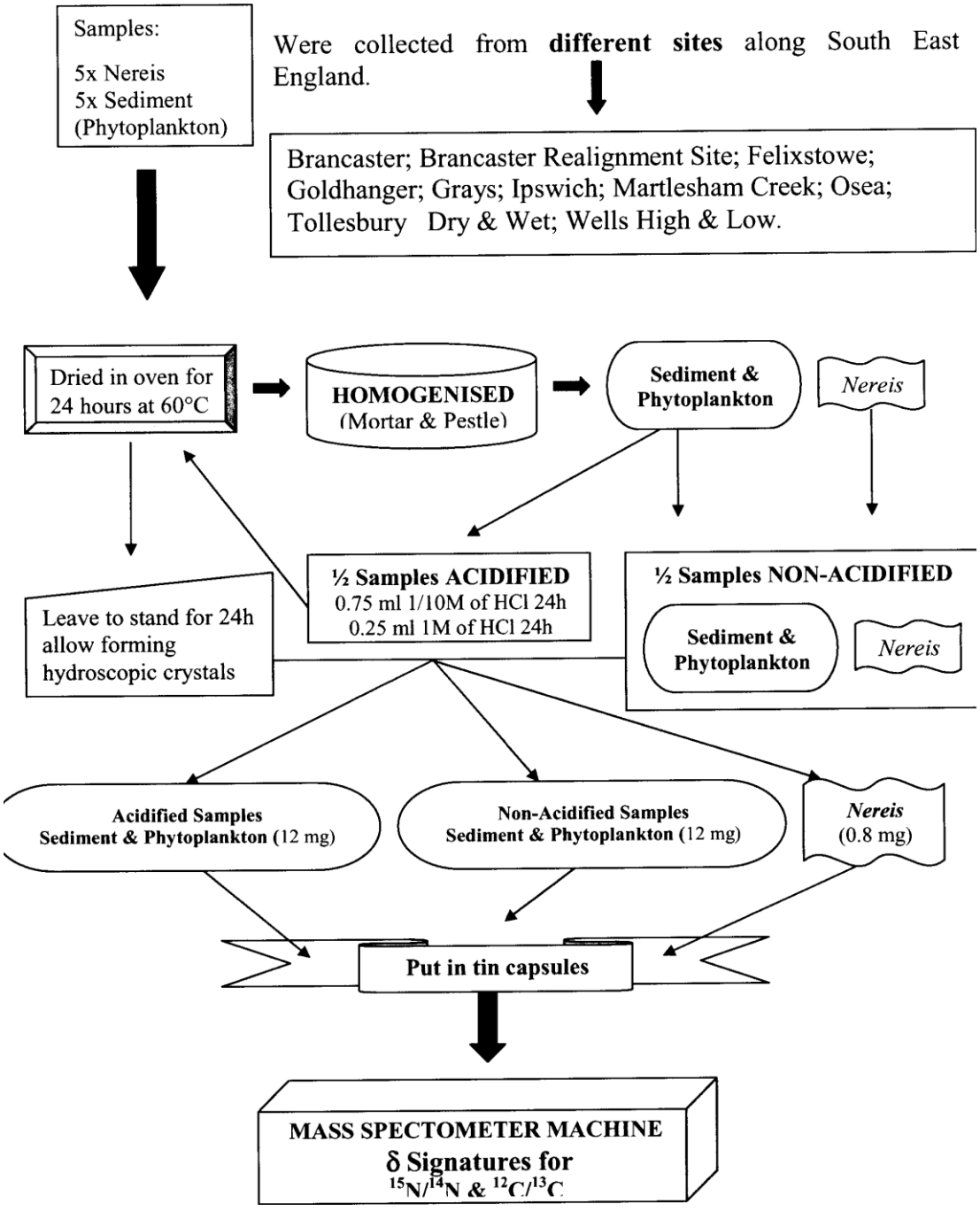
Volume of 0.1M UREA standard (ml)	0ul	5ul	10ul	40ul	70ul	100ul
Mass Urea (mg)	0	0.03	0.06	0.24	0.42	0.60
Mass Urea (ug)	0	30	60	240	420	600
Mass N (ug)	0	14	28	112	196	280
Mass C (ug)	0	6	12	48	84	120
umoles N	0	0.9995	1.9990	7.9962	13.9933	19.9904
umoles C	0	0.4995	0.9991	3.9963	6.9936	9.9908

Example 1 For nitrogen



Thus, for N the formula to calculate the mass becomes:
Mass of N = (Area (y) + 1.4948) / 0.9646

Summary of the Method:



4. Results

The figures **12 to 20** show the mean and standard error bars of the stable isotope signatures for *Nereis* (N), sediments (S) and phytoplankton (Phyt.) (when available) from each different site.

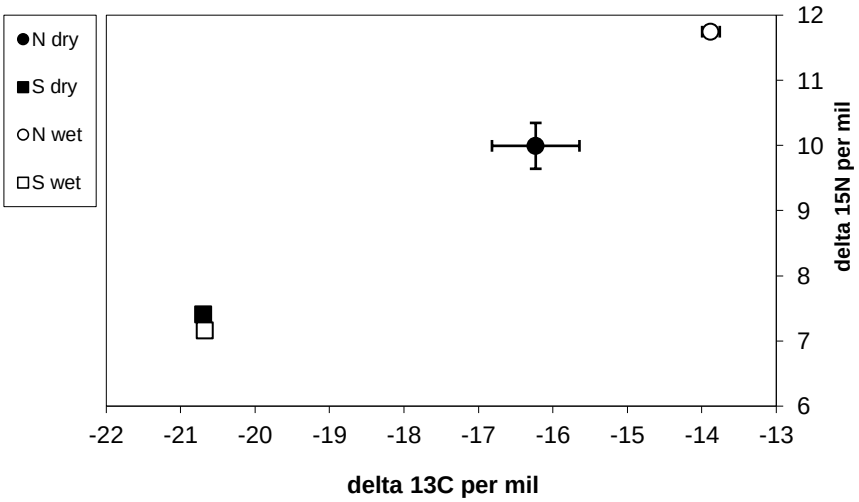


Figure 12 shows Tollesbury Dry & Wet, where no phytoplankton samples were collected. Note that the sediments from dry and wet have approximately the same isotopic signatures. *Nereis* in dry sites seems to be feeding more in the sediment samples rather than *Nereis* in wet, because these one is more further apart (more ^{13}C and ^{15}N enriched) than *Nereis* in dry, and most probably, is doing filter feeding or even feeding in something else.

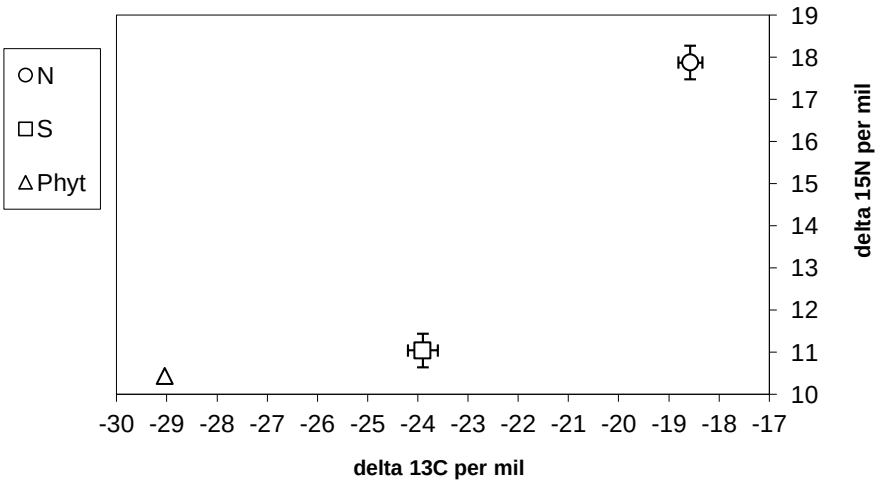


Figure 13 shows Martlesham Creek where *Nereis* seems to be feeding more at the sediment rather than the phytoplankton because δ ^{13}C & ^{15}N of Nereis is more close to isotopic signatures of sediment (more deposit feeding) rather than the phytoplankton (filter feeding).

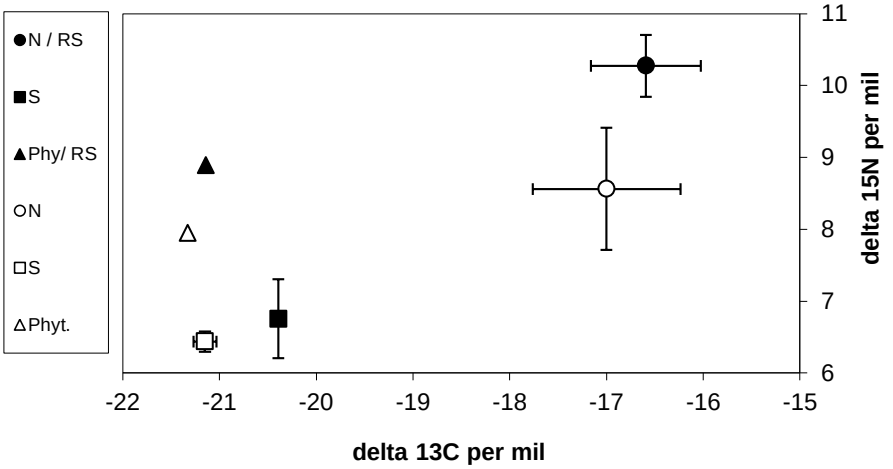


Figure 14 shows Brancaster & Brancaster Realigment Site where both the isotopic signatures of *Nereis* indicate that feeding in general is slightly more in the phytoplankton rather than in the sediments. But the sediment sample of RS seems to be more carbon enriched and less nitrogen enriched compared to RS phytoplankton.

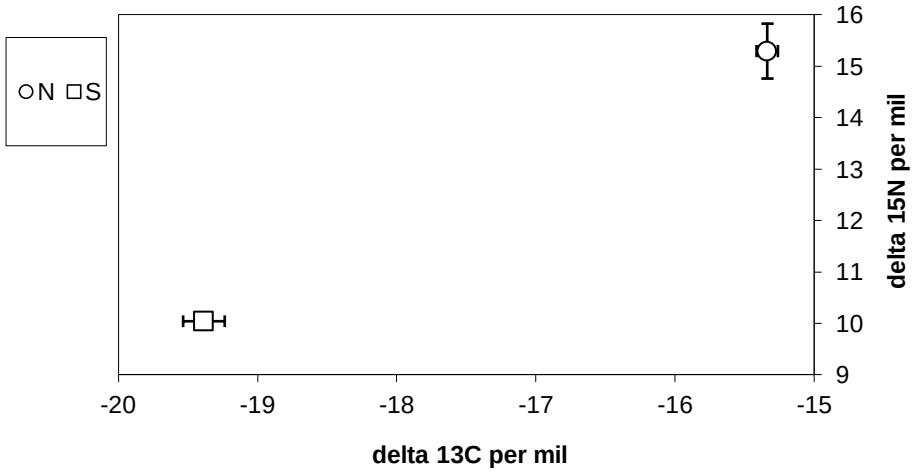


Figure 15 shows Gold Hanger where no phytoplankton samples were collected, therefore determining the exact food source is more difficult, but *Nereis* is definitely having both carbon and nitrogen enriched. Presumably phytoplankton is less nitrogen enriched than sediments; hence the enrichment of *Nereis* comes slightly more from sediment sample.

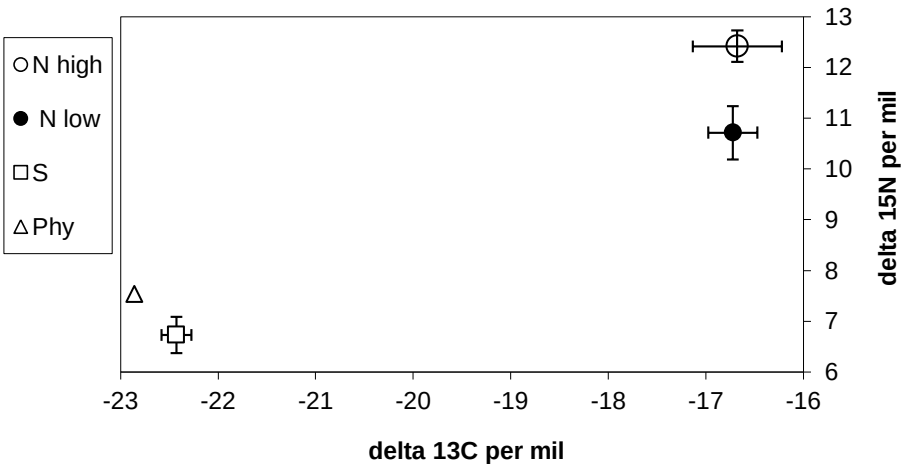


Figure 16 shows Wells High & Low where both *Nereis* from High and Low seem to be more inclined to feed in phytoplankton, but presumably it is feeding in general in both phytoplankton and sediment because there is no much difference between them (slightly higher nitrogen for phytoplankton). *Nereis* from high is more enriched in nitrogen than *Nereis* from low and that difference indicates the feeding is more inclined to phytoplankton.

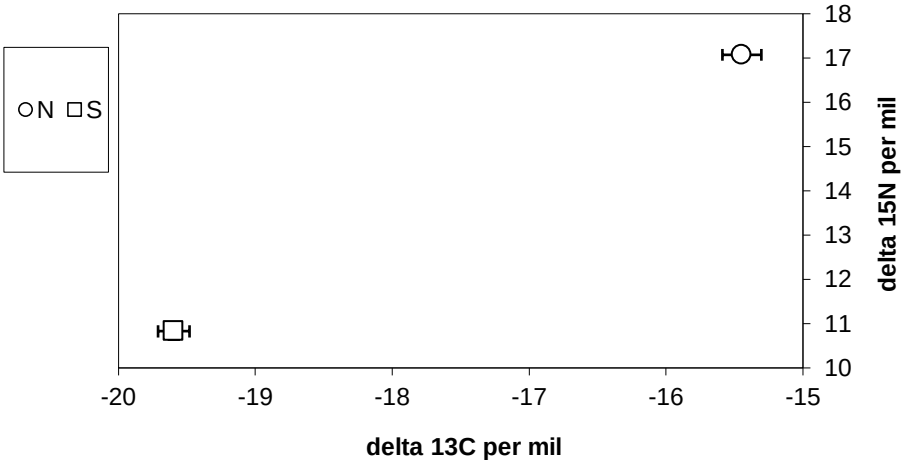


Figure 17 shows Osea where no phytoplankton sample were collected, therefore determining the exact food source is more difficult, but *Nereis* is definitely having both carbon and nitrogen enriched. Presumably phytoplankton is less nitrogen enriched than sediments; hence the enrichment of *Nereis* comes slightly more from sediment sample.

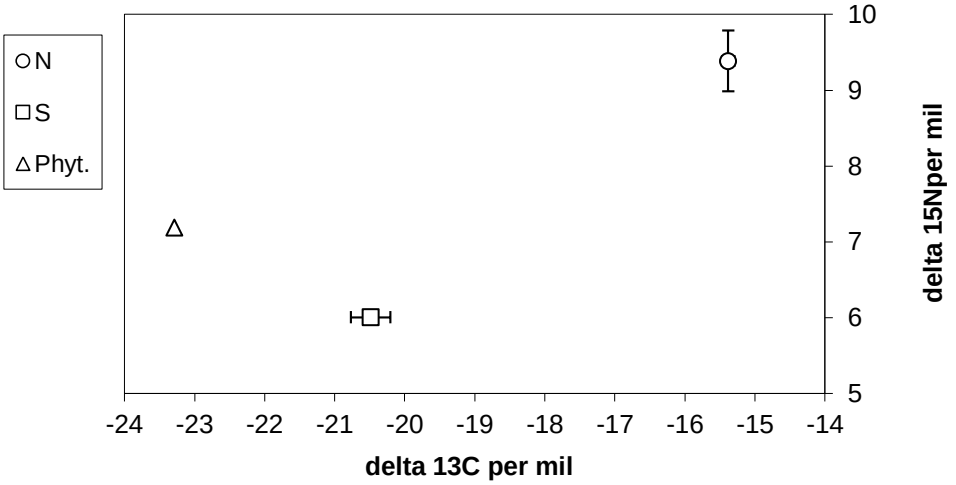


Figure 18 shows Felixstowe where it shows that feeding is more inclined to phytoplankton rather to sediment because phytoplankton is more enriched and closer to the isotopic signature of *Nereis* rather than the sediments.

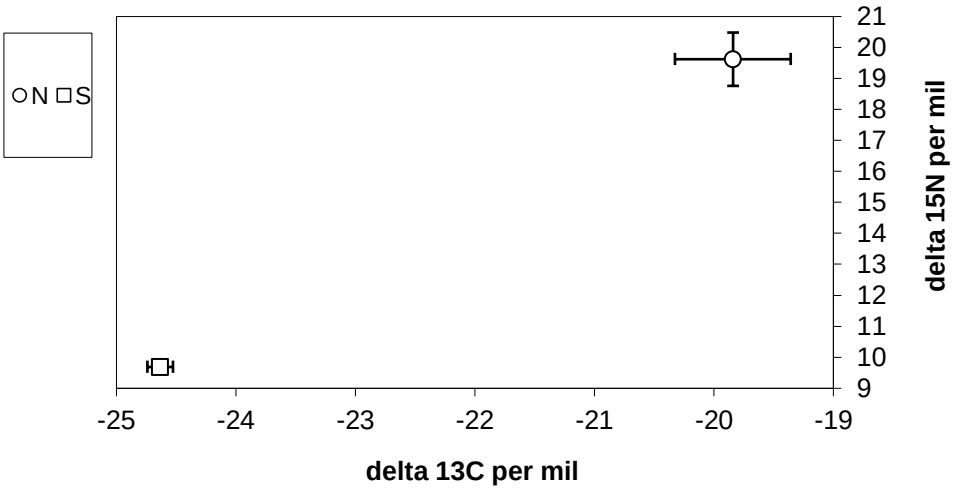


Figure 19 shows Grays where no phytoplankton sample were collected, therefore determining the exact food source is more difficult, but *Nereis* is definitely having both carbon and nitrogen enriched. Presumably phytoplankton is less nitrogen enriched than sediments; hence the enrichment of *Nereis* comes slightly more from sediment sample.

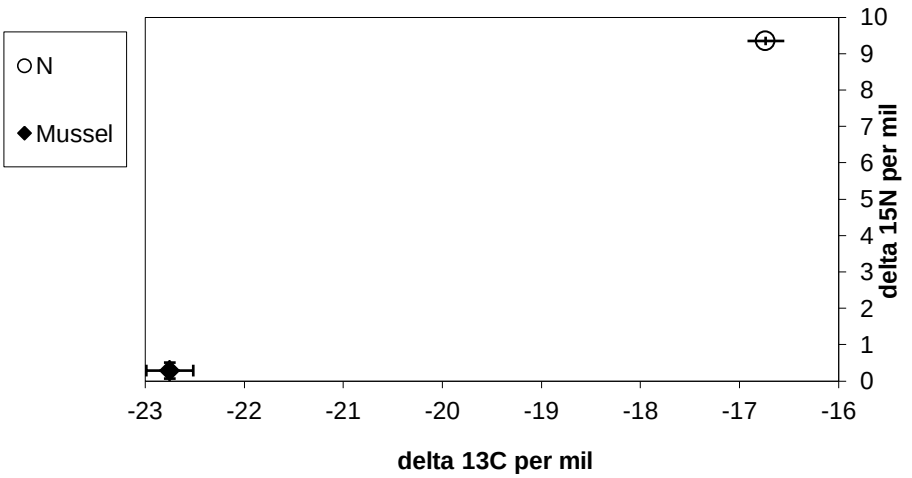


Figure 20 shows Ipswich where it illustrates the position of the isotopic signature of a mussel (filter feeder) compared to the one of Nereis (filter or deposit feeder), has it can be seen, filter feeders have a lower $\delta^{13}C$ & $\delta^{15}N$ signatures (more depleted) compared to ones of Nereis (more enriched). Unfortunately no samples of sediment and phytoplankton were able within time to be prepared and analysed, but clearly shows that the Nereis enrichment comes from sediments rather than phytoplankton.

The figures 21 to 24 show the mean and standard error bars of the stable isotope signatures from all the samples from each different site, where the Y- abscissa axis that gives the values for δ^{15} N (‰) and X-ordinate axis that gives the values for δ^{13} C (‰).

Figure 21: Mean isotopic signature (± SE) of *Nereis* from all sites

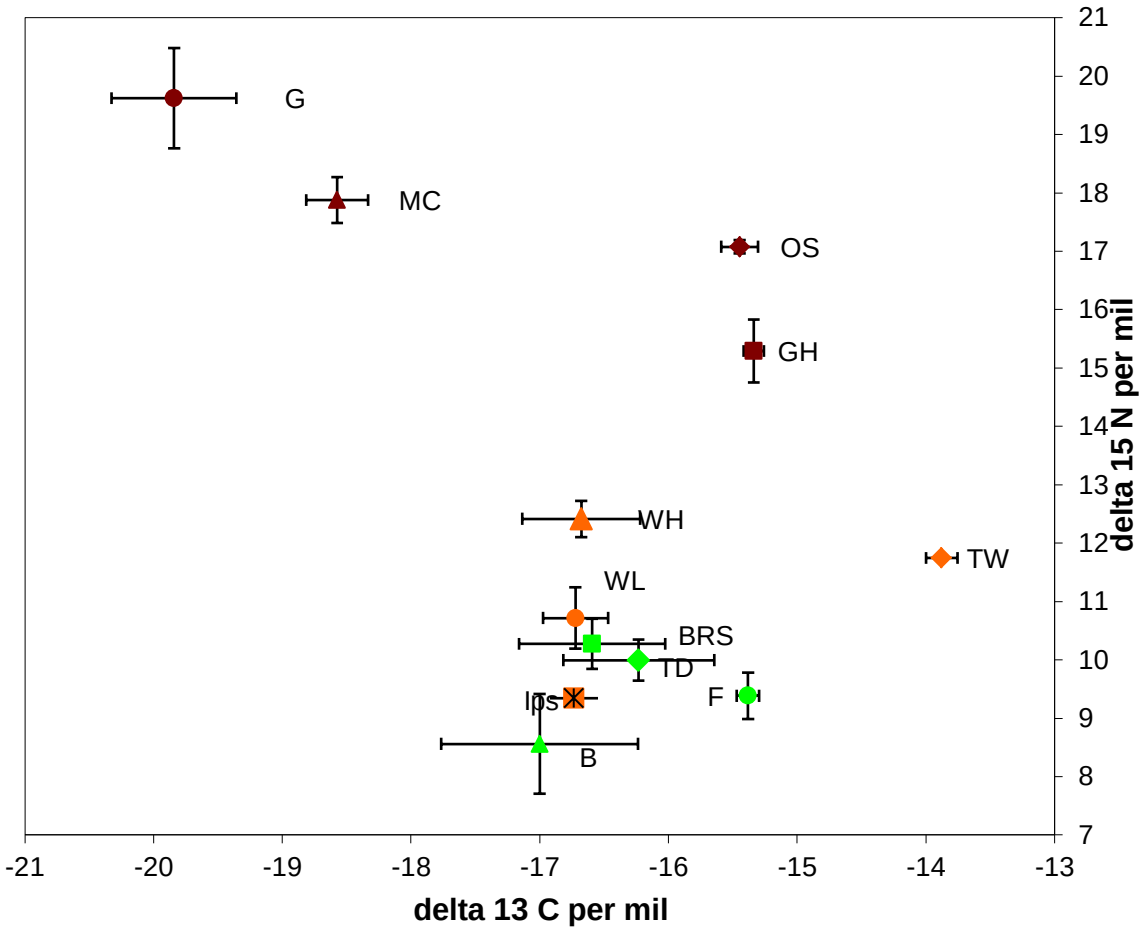


Figure 21 shows the mean isotopic signatures of *Nereis* from all sites. The green colour shows the presumed clean sites, where orange colour shows the supposed semi-polluted sites (affected by near sewage) and brown colour shows the alleged most polluted sites (close to sewage). Figure 21 shows that there are differences between the δ isotopic signatures from *Nereis* from polluted sites (brown) δ^{15} N 15 ‰ to 20 ‰ and non-polluted (green) where δ^{15} N 8 ‰ to 11 ‰, while the semi-polluted sites (orange) are between with δ^{15} N 9 ‰ to 13 ‰.

The δ^{13} C signatures of *Nereis* are all between –14 ‰ to – 18 ‰ in except for two MC- Martlesham Creek & G- Grays) where the δ^{13} C signatures ranged from –18 ‰ to –20 ‰ showing that they are more δ 13C depleted but more δ 15N enriched.

Figure 22: Mean isotopic signatures (± SE) of *Nereis* and phytoplankton from all sites

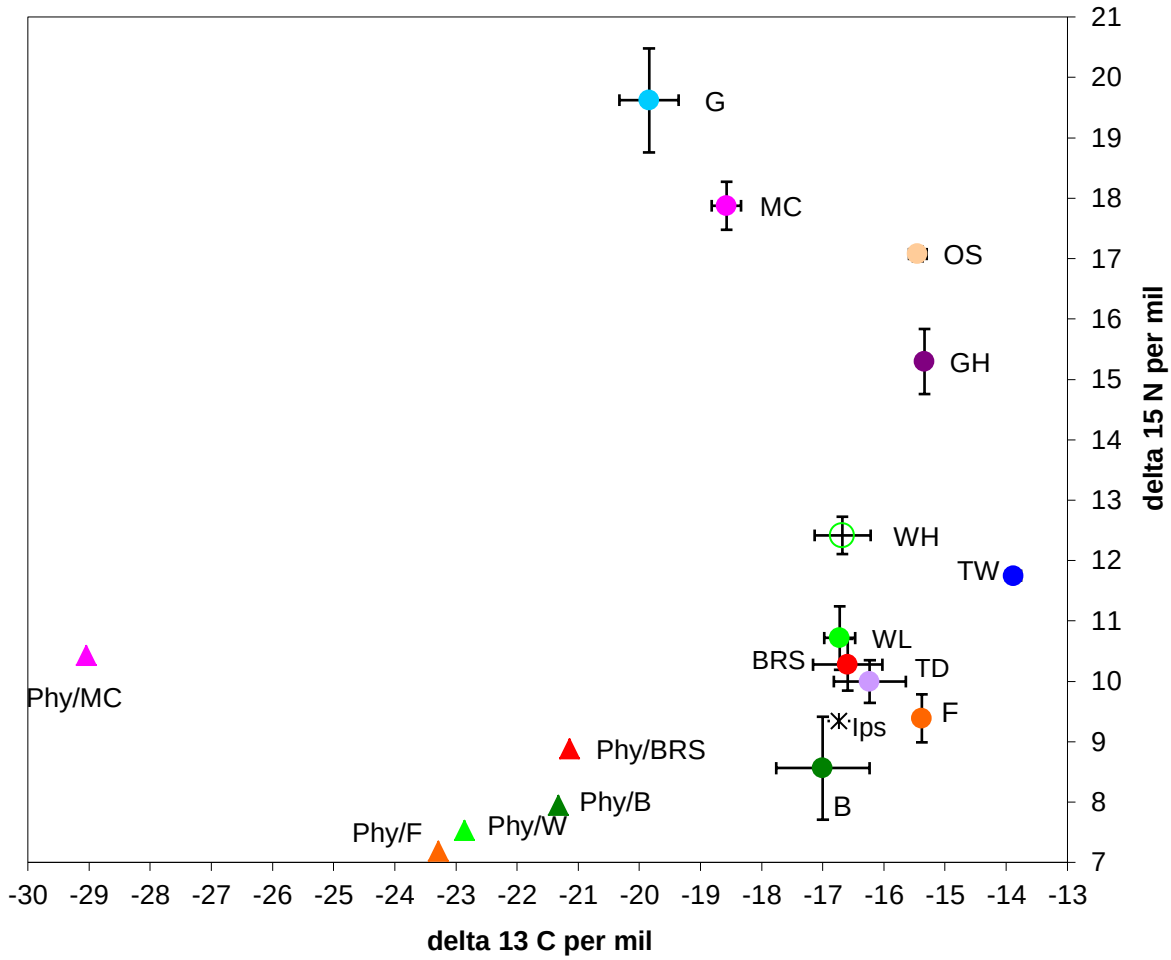


Figure 22 shows the mean isotopic signatures of *Nereis* and phytoplankton observed in all sites. The triangle shapes represent phytoplankton samples (possible food source when tide is in (Fig. 1B)) at each different site while the circles represent *Nereis* at each different site with the corresponded colour and abbreviated letter(s).

The range of δ^{15} N for all phytoplankton samples was 7 ‰ to 9 ‰ with the exception of MC-Martlesham Creek (10.4 ‰) that is more enriched in δ^{15}N. The range of δ^{13} C signatures for all phytoplankton were –20 ‰ to –24‰ and except again for Martlesham Creek (-29 ‰) where it seems to be more depleted in δ^{13}C.

Figure 23: Mean of isotopic signatures (± SE) of *Nereis* and sediments from all sites

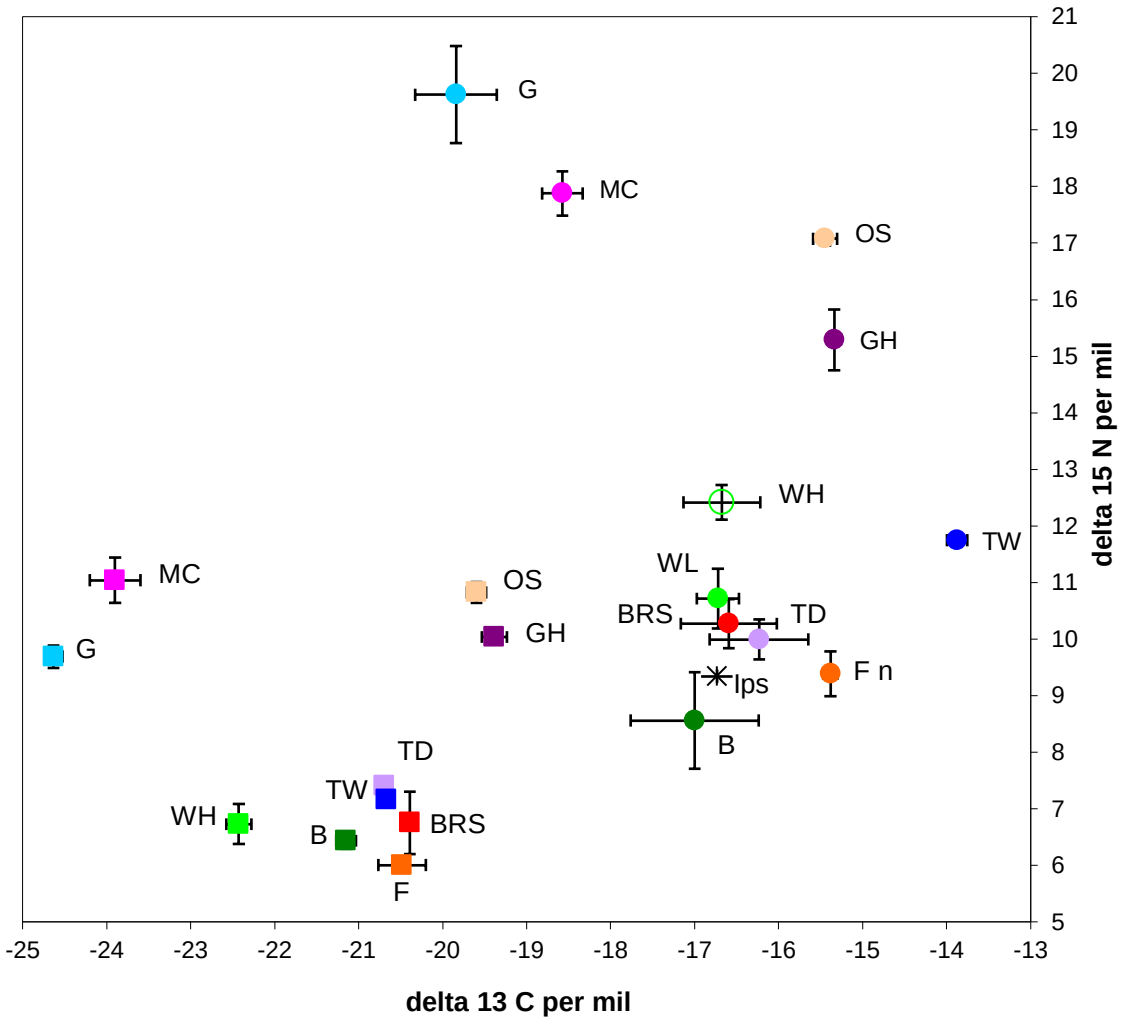


Figure 23 shows the mean isotopic signatures of *Nereis* vs. sediment observed in all sites. The square shapes represent sediment samples (possible food source when tide is in or out (Fig.1A)) from all sites while the circles represent *Nereis* at each different site with the corresponded colour and abbreviated letter(s).

The range of δ^{15} N for the sediments were 6 ‰ to 8 ‰ with the exceptions of GH-Gold Hanger, OS-Osea, MC-Martlesham Creek and G-Grays where δ^{15} N were 9 ‰ to 11‰, while the range for δ^{13} C signatures for all sediments were –19 ‰ to –22 ‰, with the exceptions of Grays and Martlesham Creek which ranged from –24 ‰ to –25 ‰ showing that is more depleted in δ^{13}C and more enriched in δ^{15}N.

5. Discussion

To understand and to interpret the data, there is the need to look at how carbon and nitrogen stable isotopes circulate within biological systems. The carbon cycle is a process by which carbon from atmospheric carbon dioxide (CO_2) enters the biosphere. This then circulates within the biosphere as organic carbon, and eventually returns to the atmosphere as CO_2. Thus, carbon enters the biosphere by photosynthetic fixation of CO_2 into organic compounds and is returned to atmosphere as CO_2 formed mostly by respiration of living organisms. This also occurs by the burning of wood and fossil fuels.

Carbon isotopic compositions of animals reflect those of the diet within 1‰ enrichment in the animal relative to its diet (**Fig. 2** Peterson and Fry, 1987 from Latjha & Michener 1994). There are several possible processes that might contribute to enrichment: (a) such as preferential loss of $^{12}CO_2$ during respiration; (b) preferential uptake of ^{13}C compounds during digestion; or (c) metabolic fractionation during synthesis of different tissue types (DeNiro & Epstein, 1978; Rau et al., 1983; Tiezen et al., 1983; Fry et al., 1984).

However, there is a need for awareness of isotopic variations in different tissues within an organism, as well as the different rates of tissue turnover when an organism is selectively feeding. This can have implications for estuarine systems, where there is more than one potential food source. Because of the large differences in $\delta\,^{13}C$ of organic matter from marine, terrestrial and marsh environments, stable isotopes provide a useful tool to trace the transport of material in the water column and sediments. Most of particulate organic carbon (POC) in estuaries is derived from photosynthesis. Usually, the organic matter from terrestrial sources (C_3 plants) is relatively depleted in ^{13}C, where aquatic Macrophytes and C_4 marsh grasses are enriched in ^{13}C, and phytoplankton is intermediate (Fry & Sherr, 1984). Organic matter from primary production in marine, freshwater, marsh and terrestrial ecosystems has a $\delta\,^{13}C$ range of –35 to -5‰ (**Fig. 24** from Latjha & Michener 1994). Where in estuaries the $\delta\,^{13}C$ of POC range is approximately –33 to –18 ‰, the reported values normally indicate that the primary sources of POC are terrestrial organic matter and phytoplankton, and the importance of these two main sources depends on sample location and in the hydrodynamics of the estuary. However

seagrasses, macroalgae and C4 marsh plants are not usually major contributors to estuarine POC.

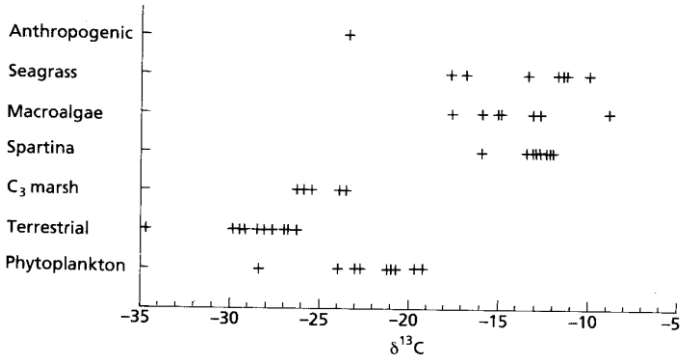


Figure 24 shows the ranges of stable carbon isotope ratios ($\delta^{13}C$) of potential carbon sources for bacteria. (From Latjha & Michener 1994)

Primary producers in seawater fix inorganic carbon as CO_2 and/or HCO_3^- (Fogel & Cifuentes, 1993). The isotope ratio of dissolved inorganic carbon (DIC) is also important for tracing carbon sources because this ratio strongly influences the value of the primary producer. The $\delta^{13}C$ of DIC in aquatic ecosystems can vary spatially and seasonally within an estuary. Therefore stable isotope ratios of DIC in estuaries range from –29 to +2‰ (From Latjha & Michener 1994).

The nitrogen cycle is a process by which nitrogen circulates between the atmosphere and the biosphere. Atmospheric elemental nitrogen (N_2) is converted by a few groups of soil and aquatic bacteria into inorganic nitrogenous compounds, primarily ammonia, by the process of nitrogen fixation. These inorganic compounds are then incorporated into plants and bacteria and then into animals, with the synthesis of complex nitrogen-containing organic molecules in their tissues. Organic nitrogen-containing compounds are subsequently broken down by bacteria and fungi (ammonification and nitrification). This generates inorganic nitrogen compounds such as ammonia, nitrites and nitrates, which may be used by plants as nutrients, or may be converted to elemental nitrogen or nitrous oxide by certain bacteria (denitrification) thus releasing nitrogen to the atmosphere. This cycle also incorporates non-biological exchanges of nitrogen between atmosphere and biosphere as in the precipitation of inorganic nitrogen compounds in rainwater, and the fixation of atmospheric nitrogen by lightning.

However, field studies show an average of 3.2‰ enrichment in animal $\delta\ ^{15}N$ versus diet (**Fig. 3**), which is reflected as a trophic level effect in food web studies. Nitrogen is recognized as an essential and often limiting element in primary production. The natural cycling of nitrogen in the environment has been extensively changed by human activities. It is commonly believed that application of fertilizers and /or the influence of animal or sewage wastes have contributed to the abundance of nitrate (NO_3^-) in ground water. However, nitrogen (N_2) in soils is eventually derived from two sources, atmospheric nitrogen and geological materials. The majority of nitrogen in the biosphere and hydrosphere is derived from atmospheric gas via biological nitrogen fixation (**Fig 25** Step1), although in some environments larger portions derive from NO_3^- or ammonium (NH_4^+) deposition from anthropogenic sources (Aber et al., 1989).

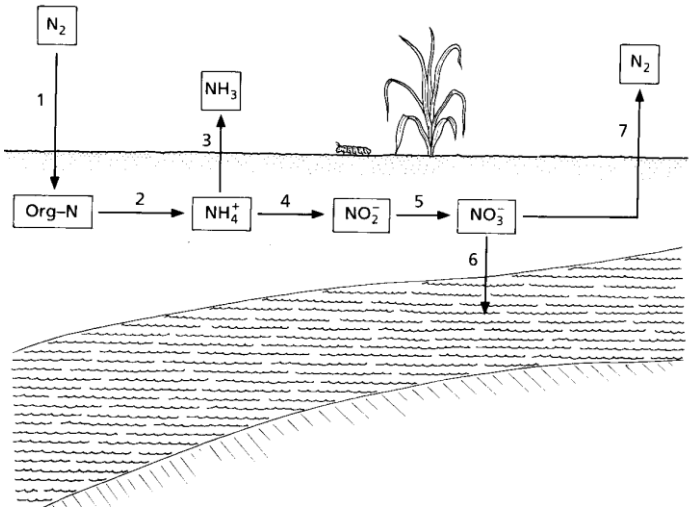


Figure 25 shows a simplified diagram of the nitrogen cycle in soils and ground waters. (From Latjha & Michener 1994)

The atmospheric N_2 is also used to produce NH_4^+ for fertilizers, and this process is increased once incorporated in soils. Organic matter is utilized as a substrate for microbial growth in many reactions. Step 2 in (**Fig 25**) shows that mineralization is a process whereby NH_4^+ is generated from organic matter during decomposition. In step 3, once generated or applied as fertilizer, NH_4^+ is inclined to volatilisation under conditions of high pH into ammonia (NH_3^+), or oxidation to nitrite (NO_2^-) in step 4 and NO_3^- in step 5, by microbial nitrification. Step 6 shows when nitrate is readily soluble, it is not as reactive with the soil organic matter complex as NH_4^+, and is easily transported by the movement of water through the

soil, and in step 7 it can be lost to soils or ground waters through denitrification. This process requires anoxic or near anoxic conditions to result in the production of N_2, nitrogen oxide (N_2O) and other nitrogen oxide gases. Denitrification is shown to occur in soils, phreatic water and sometimes in aquifers. This is the mechanism that completes the nitrogen cycle by returning N_2 to the atmosphere. Each step in the nitrogen cycle may result in isotopic fractionation (Shearer et al., 1974a). However, isotopic fractionation factors for nitrate or ammonium uptake by microbes or aquatic algae when supplied with inorganic nitrogen, are in the range of –10 to –20 ‰ (Macko et al., 1987). Three sources of nitrate to surface and ground waters have been recognized as major contributors to ground water systems: (a) fertilizer nitrogen; (b) natural soil-derived nitrate; and (c) animal waste or sewage-derived nitrate, other sources such as precipitation, nitrogen fixation and geological materials may be significant at specific locations but is in general not considered important (From Latjha & Michener 1994). Sewage transported into aquatic environments has characteristic isotope signatures that make resolution and quantification possible and can also be successfully distinguished from marine or freshwater productivity on the basis of stable isotope ratios.

Wong & Sackett (1978) found that marine phytoplankton species would differ in their metabolic pathways, leading to a range of delta (δ13C algae vs. HCO_3^-) of -22.1‰ to -35.5‰ for 17 species in laboratory cultures. The abundance and various forms of inorganic nitrogenous nutrients affect nitrogen isotope ratios in phytoplankton. There are two types of organic matter; rapidly sinking particles (usually made up of faecal pellets and marine snow), and slowly sinking particles that are generally decomposed and remineralized within the euphotic zone (Saino & Hattori, 1987). It is very difficult to obtain clean samples of phytoplankton PON for analysis. Therefore, PON samples usually include mix detritus, bacteria, phytoplankton and microzooplankton (Altabet & McCarthy, 1985).

However, it has been established that carbon and nitrogen pass from one trophic level to the next one and the heavier partitions of the isotope tend to be assimilated at a higher rate than the lighter isotopes. This rate usually leads to a slight enrichment (increase) in the animal relative to its diet (**Fig. 2**), towards the heavier

isotope by 0.5-2 ‰ for carbon (^{13}C) and 3-5 ‰ for nitrogen (^{15}N) per trophic level (Peterson and Fry, 1987. Eggers & Jones, 2000).

It is known that *Nereis* feed from a variety of food sources. However in this study, two particular food sources (sediments and phytoplankton) were concentrated on. These potential food sources fit the enrichment relationship pattern established by Peterson and Fry 1987 (**Fig. 2**). The results clearly show that there is enrichment of nitrogen ($\delta^{15}N$) from sediments and phytoplankton in all sites. However, this was actually higher in some sites studied, (over 5 ‰). For example, in Martlesham Creek with 7 ‰ (**Figure 13, 22& 23**), Grays with 10 ‰ (**Figure 19 & 23)**, Wells High with 6 ‰ (**Figure 16, 22 & 23**) and Osea with 6 ‰ (**Figure 17 & 23**).

In relation to carbon ($\delta^{13}C$), the results also show that there is enrichment in all sites, in fact it is greater than 2 ‰ in all the sites studied.

In the clean and less polluted sites studied, *Nereis* seems to be feeding on both sediments and phytoplankton. The results show that the feeding on phytoplankton is slightly higher than the sediments (**Figure 12** shows Nereis at Tollesbury-wet site, is using more filter feeding method compared to deposit feeding, which seems to be more used in Tollesbury-dry site; **Figure 14** Brancaster & BRS; **Figure 16** Wells-High rather than Wells-Low; & **Figure 18** Felixstowe). The $\delta^{13}C$ and $\delta^{15}N$ isotope signatures of sediments and phytoplankton are reasonably the same in comparison to the $\delta^{13}C$ and $\delta^{15}N$ tissue enrichment of *Nereis* (**Figure 22 & 23**). However, phytoplankton is slightly depleted in $\delta^{13}C$ and slightly enriched in $\delta^{15}N$ in comparison to the signatures of the sediments samples (**Figure 22 & 23**). Thus phytoplankton, on clean sites, provides a slightly richer food source for *Nereis* than sediment.

In the polluted sites studied, *Nereis* seems to be feeding significantly more on sediments rather than phytoplankton. This is because the $\delta^{13}C$ and $\delta^{15}N$ isotope signatures of sediments are much closer to the ones corresponding to the tissue enrichment of *Nereis* rather than the phytoplankton isotope signatures. Phytoplankton, seems to be more depleted (distant) in $\delta^{13}C$ and $\delta^{15}N$ compared to the isotope signatures of sediments (**Figure 13** for Martlesham Creek as an example).

Therefore, enrichment of sites with anthropogenic sources of nitrogen, particularly sewage, have been shown to increase $\delta^{15}N$ values as also seen in many

marine studies (Kikuchi & Wada, 1996. Riera, 1998. Riera et al., 2000. Wadron et al., 2001). The results show that Gold Hanger, Osea, Martlesham Creek and Grays are the most enriched habitats (most polluted sites where $\delta^{15}N$ signatures are in between 15 ‰ to 20 ‰), rather than the least polluted sites, Brencaster, Felixstowe, Ipswich, Tollesbury Dry, Brencaster Realigment Site and Wells Low (least polluted sites where $\delta^{15}N$ signatures are in between 8 ‰ to 11 ‰ in **Figure 21 & 23**). The sites in between are Tollesbury Wet & Wells High (sites affected by sewage where $\delta^{15}N$ signatures are in between 12 ‰ to 13 ‰). The isotopic signatures for *Nereis* tested on polluted sites are, on average, at least 6 ‰ of $\delta^{15}N$ higher than the organisms tested on least polluted sites, where on average were at least 3 ‰ $\delta^{15}N$. Consequently, this supports the theory that anthropogenic inputs of nitrogen, particularly in form of sewage, are assimilated into food webs and this affects all organisms present at that particular site or ecosystem. For *Nereis*, sewage is a form of nutrition where there are important implications to estuarine conservation; especially for those most sewage affected sites. It was clearly visible, when collecting samples, during this studied, that there was a higher number of *Nereis* (m^2), in sewage polluted sites compared to other cleaner sites (least affect by sewage).

Reduced nitrogen outputs will reduce the nitrogen input into the food webs. This could reduce the numbers of *Nereis* and would effectively help to establish more surface dwelling algae and also the pioneer species. A more stable sediment surface would be achieved, encouraging the pioneer species to thrive and lead to mature saltmarsh development. However this would decrease invertebrate prey densities and consequently, could cause a decline in the migrating bird populations in the short term. Nevertheless, through the development of a more mature saltmarsh, the initial loss of nutrition will be replaced by the increased primary production within the estuaries.

6. Conclusion

The data supports the hypothesis formulated in the beginning of this paper, as this study shows, that there is a difference in the nitrogen isotopic signatures between sewage-affected sites and those of clean sites. This indeed reflects the greater dependence on deposit feeding rather than filter feeding that is typically found in clean sites. The differences in the δ^{15} N signatures are due to anthropogenic inputs, particularly in the form of sewage. This study also shows that *Nereis* in polluted sites are not feeding as a suspension feeder but instead, are feeding mainly on the stabilising mud surface sediments. These also include everything that is in and on the sediment such as microphytobenthos, and seedlings from pioneer sea grasses species. The results emphasize the importance of *Nereis* behaviour as an agent of erosion in saltmarshes, especially in areas most affected by sewage pollution. Hence, these results show the importance of anthropogenic sources of nutrition to estuarine ecosystems and the relevance of this data should be considered in respect to conservation and saltmarsh management.

7. Acknowledgements

I am extremely grateful to Dr. R.G. Hughes and would like to thank him for assistance and guidance in carrying out this research project and particularly, for his belief in me and the reassurance and encouragement that he gave me over my three years at Queen Mary University. I would also like to thank Jackie Hill for all her help and guidance in preparing samples for, and using the Mass Spectrometer machine. My thanks also go out to all the members of staff in the school of biological science who have helped in the production of this project.

I would also like to thank my parents, family and friends, for all the strength and support they have given to me over the past three years.

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9. Appendix

Isotopic signatures for all samples after mass spectrometer machine with values not adjusted to international standards (I.S.)

d 15N/14 N	Site	d 13C/12C
8.979	TD n	-15.538
9.995	TD n	-16.472
11.01	TD n	-16.685
7.34	TD s	-20.54
7.354	TD s	-20.71
7.531	TD s	-20.835
11.536	TW n	-13.819
11.956	TW n	-13.94
11.752	TW n	-13.869
7.295	TW s	-20.591
7.111	TW s	-20.633
7.077	TW s	-20.797
18.345	MC n	-17.985
17.738	MC n	-18.413
17.547	MC n	-19.324
11.204	MC s	-23.204
10.458	MC s	-24.596
11.458	MC s	-23.905
10.428	Phy/MC	-29.04
9.144	BRS n	-16.121
10.715	BRS n	-16.201
10.964	BRS n	-17.456
6.706	BRS s	-20.413
6.828	BRS s	-21.33
6.724	BRS s	-19.429
8.887	Phy/BRS	-21.137
9.193	B n	-15.716
7.04	B n	-18.611
9.45	B n	-16.673
6.58	B s	-20.896
6.527	B s	-21.38
6.205	B s	-21.176
7.944	Phy/B	-21.325
15.43	GH n	-16.184
15.286	GH n	-15.488
15.162	GH n	-14.339
9.739	GH s	-19.156
10.179	GH s	-19.463
10.201	GH s	-19.537

d 15N/14 N	Site	d 13C/12C
12.431	WH n	-16.873
11.618	WH n	-16.07
13.202	WH n	-17.089
10.323	WL n	-16.065
10.64	WL n	-17.763
11.187	WL n	-16.339
6.524	WH s	-22.169
7.026	WH s	-21.987
6.638	WH s	-23.126
7.528	Phy/W/B	-22.857
16.898	OS n	-15.627
16.971	OS n	-15.231
17.359	OS n	-15.48
10.598	OS s	-19.482
10.923	OS s	-19.348
10.957	OS s	-19.96
9.293	F n	-15.776
9.303	F n	-15.784
9.562	F n	-14.584
6.274	F s	-20.302
6.301	F s	-20.635
5.443	F s	-20.515
7.19	Phy/F	-23.283
18.72	G n	-21.543
19.768	G n	-18.781
20.379	G n	-19.199
9.786	G s	-24.803
9.809	G s	-24.859
9.479	G s	-24.242
9.124	Ips n	-16.524
9.193	Ips n	-16.936
9.715	Ips n	-16.74
0.749	Ips M	-23.093
0.145	Ips M	-22.341
-0.04	Ips M	-22.817

Mean of all samples **Standard Deviation from all samples**

With adjustments with I.S.

Mean n			STDev	
9.994666667	TD n	-16.23166667	1.015500041TD n	0.610100265
7.408333333	TD s	-20.695	0.106462826TD s	0.148070929
11.748	TW n	-13.876	0.210028569TW n	0.06080296
7.161	TW s	-20.67366667	0.117285975TW s	0.108854643
17.87666667	MC n	-18.574	0.416680133MC n	0.683864753
11.04	MC s	-23.90166667	0.519780723MC s	0.696005987
10.428	Phy/MC	-29.04		
			0.98678282BRS n	0.748737827
10.27433333	BRS n	-16.59266667	0.065858434BRS s	0.950696762
6.752666667	BRS s	-20.39066667		
8.887	Phy/BRS	-21.137	1.323477616B n	1.474941694
			0.202944163B s	0.242992455
8.561	B n	-17		
6.437333333	B s	-21.15066667	0.13412432GH n	0.931722598
7.944	Phy/B	-21.325	0.260617216GH s	0.202025576
15.29266667	GH n	-15.337	0.792092798WH n	0.536939786
10.03966667	GH s	-19.38533333	0.437072458WL n	0.911597133
			0.263167881WH s	0.611867905
12.417	WH n	-16.67733333		
10.71666667	WL n	-16.72233333	0.247788216OS n	0.200177421
6.729333333	WH s	-22.42733333	0.198184258OS s	0.321710014
7.528	Phy/W	-22.857		
			0.152502459F n	0.690522507
17.076	OS n	-15.446	0.487759162F s	0.168650526
10.826	OS s	-19.59666667		
			0.839037742G n	1.488718912
9.386	F n	-15.38133333	0.184245307G s	0.341210101
6.006	F s	-20.484		
7.19	Phy/F	-23.283	0.323142383Ips1	0.20608089
19.62233333	G n	-19.841	0.412626142IpsM	0.3804068
9.691333333	G s	-24.63466667		
9.344	Ips n	-16.73333333		
0.284666667	Ips M	-22.75033333		

Standard Error of samples from all sites with adjustments with IS

SE- N		SE- C
0.586299222	TD n	0.352241552
0.061466341	TD s	0.085488791
0.121260051	TW n	0.035104606
0.067715089	TW s	0.062847258
0.240570387	MC n	0.394829499
0.30009554	MC s	0.401839244
0.569719327	BRS n	0.432283986
0.038023385	BRS s	0.548885031
0.764110158	B n	0.851557984
0.117169867	B s	0.140291759
0.077436713	GH n	0.537930293
0.15046742	GH s	0.116639521
0.45731499	WH n	0.31000233
0.252343901	WL n	0.52631085
0.151940047	WH s	0.3532621
0.143060593	OS n	0.115572488
0.114421735	OS s	0.185739363
0.088047336	F n	0.398673356
0.281607883	F s	0.097370427
0.484418666	G n	0.859512265
0.106374078	G s	0.196997744
0.186566342	Ips1	0.118980857
0.238229814	IpsM	0.219627968