



CARDIFF UNIVERSITY

School of Biosciences

**GROWTH, METABOLISM, ULTRASTRUCTURE AND
CHEMOTHERAPY OF *SPIRONUCLEUS VORTENS***

Coralie Millet

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September 2009

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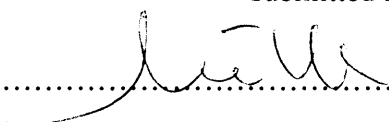
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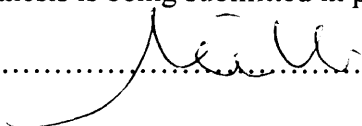
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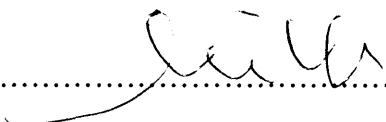
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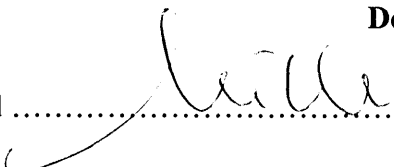
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GROWTH, METABOLISM, ULTRASTRUCTURE AND CHEMOTHERAPY OF *SPIRONUCLEUS VORTENS*

ABSTRACT	1
CHAPTER 1 : GENERAL INTRODUCTION	2
CHAPTER 2 : <i>IN VITRO</i> CULTURING AND GROWTH OF <i>SPIRONUCLEUS VORTENS</i>	21
2. 1 ABSTRACT	21
2. 2 INTRODUCTION	22
2. 3 MATERIAL & METHODS	25
2. 4 RESULTS	28
2. 4. 1 Optimization of growth conditions	28
2. 4. 2 Optimizations of conditions of cryopreservation	29
2. 4. 3 Axenisation of cultures	29
2. 4. 4 Growth curves	29
2. 5 DISCUSSION	32
2. 6 REFERENCES	36
CHAPTER 3: COMPARATIVE STUDY OF PHAGOCYTOSIS IN <i>SPIRONUCLEUS VORTENS</i> AND <i>GIARDIA INTESTINALIS</i>	41
3. 1 ABSTRACT	41
3. 2 INTRODUCTION	42
3. 3 MATERIAL & METHODS	43
3. 4 RESULTS	47
3. 4. 1 Flow cytometry	47
3. 4. 2 Confocal scanning laser microscopic assay of quantum dots uptake	48
3. 4. 3 Fluorescent in situ hybridisation assay for phagocytosis of live bacteria	50

3. 5 DISCUSSION	51
3. 6 REFERENCES	53
CHAPTER 4: THE DIPLOMONAD FISH PARASITE <i>SPIRONUCLEUS VORTENS</i> PRODUCES H₂	58
4. 1 ABSTRACT	58
4. 2 INTRODUCTION	59
4. 3 MATERIAL & METHODS	61
4. 4 RESULTS	63
4. 2. 1 Gas metabolism	63
4. 2. 2 Effect of inhibitors	64
4. 5 DISCUSSION	66
4. 6 REFERENCES	68
CHAPTER 5: CARBOHYDRATE AND AMINO ACID METABOLISM IN <i>SPIRONUCLEUS VORTENS</i>	76
5. 1 ABSTRACT	76
5. 2 INTRODUCTION	77
5. 3 MATERIAL & METHODS	78
5. 4 RESULTS	82
5. 4. 1 Influence of substrate on gas metabolism	82
5. 4. 2 Identification of products of glucose and endogenous metabolism by ¹ H NMR	84
5. 4. 3 Identification of products of glucose metabolism by ¹³ C NMR	87
5. 4. 4 Influence of substrates on growth of <i>Spiroucleus vortens</i>	89
5. 4. 5 Amino acid metabolism of <i>Spiroucleus vortens</i>	91
5. 4. 5. a Analysis of free amino acid concentrations in conditioned culture medium	91
5. 4. 5. b Analysis of total amino acid concentration in hydrolysate of conditioned culture medium	94
5. 4. 6 DETECTION OF NO PRODUCTION	96
5. 5 DISCUSSION	97

5. 6 REFERENCES	101
CHAPTER 6: <i>SPIRONUCLEUS VORTENS</i> HAS BOTH A HIGH TOLERANCE AND A HIGH AFFINITY FOR O₂	106
6. 1 ABSTRACT	106
6. 2 INTRODUCTION	107
6. 3 MATERIAL & METHODS	108
6. 4 RESULTS	111
6. 4. 1 O ₂ Affinity and inhibition threshold of <i>Spironucleus vortens</i>	111
6. 4. 2 Products of glucose metabolism under various O ₂ tensions	111
6. 5 DISCUSSION	114
6. 6 REFERENCES	119
CHAPTER 7: EFFECTS OF GARLIC AND ALLIUM-DERIVED PRODUCTS ON THE GROWTH AND METABOLISM OF <i>SPIRONUCLEUS VORTENS</i>	123
7. 1 ABSTRACT	123
7. 2 INTRODUCTION	124
7. 3 MATERIAL & METHODS	128
7. 4 RESULTS	130
7. 4. 1 Effect of freeze-dried garlic on metabolism & growth of <i>Spironucleus vortens</i>	130
7. 4. 2 Effect of allicin on metabolism & growth of <i>Spironucleus vortens</i>	133
7. 4. 3 Effect of ajoene on growth of <i>Spironucleus vortens</i>	136
7. 4. 4 Effect of a mixture of dithiins and thiosulfinates on growth of <i>Spironucleus vortens</i>	138
7. 4. 5 Effect of metronidazole on metabolism & growth of <i>Spironucleus vortens</i>	140
7. 5 DISCUSSION	144
7. 6 REFERENCES	149

CHAPTER 8: BOTH MITOSOME AND HYDROGENOSOME-LIKE ORGANELLES IN THE DIPLOMONAD PARASITE <i>SPIRONUCLEUS VORTENS</i>	160
8. 1 ABSTRACT	160
8. 2 INTRODUCTION	161
8. 3 MATERIAL & METHODS	163
8. 4 RESULTS	167
8. 4. 1 Snap-freeze cryo-sectioning and standard TEM	167
8. 4. 2 Analysis of autofluorescence spectrum	176
8. 4. 3 Confocal scanning laser microscopy of cells incubated with cationic fluorophores	184
8. 4. 4 Immunofluorescent labelling of <i>Spiroucleus vortens</i>	191
8. 5 DISCUSSION	202
8.5. 1 Presence of putative mitochondria	202
8. 5. 2 Presence of putative hydrogenosomes	203
8.5. 3 Evolutionary outlooks	206
8. 6 REFERENCES	208
CHAPTER 9: CONCLUDING COMMENTS	218
PUBLICATIONS	226
APPENDIXES	227
APPENDIX 1: Preliminary data on relative prevalence of <i>Spiroucleus barkhanus</i> in British and Norwegian grayling (<i>Thymallus thymallus</i>) and reports on <i>in vitro</i> cultivation attempts of <i>Spiroucleus torosa</i>	227
APPENDIX 2: Amplification of the SSU 18S rDNA fragment of <i>Spiroucleus vortens</i> using a primer developed for <i>Spiroucleus barkhanus</i>	232

APPENDIX 5. 4. 4: Output of automated optical density monitoring of growth in diluted culture medium complemented with various substrates	236
APPENDIX 5. 4. 5: Changes in free amino acid concentration in conditioned Keister's modified TY-I-S33 culture medium	237
A. After 4 days of growth	237
B. After 6 days of growth	238
APPENDIX 5. 4. 6: Changes in total amino acid concentration in hydrolysate of conditioned culture medium after 4 days of growth	239
APPENDIX 7: Output of automated optical density monitoring of <i>S. vortens</i> in Keister's modified culture medium upon addition of inhibitors	240
A. Metronidazole	240
B. Freeze-dried garlic	241
C. Allicin	242
D. Ajoene	243
E. Mixtures of dithiins and ajoene-free thiosulfinates	244

ABSTRACT

Spiroucleus species are anaerobic, flagellated protozoa, which can be either parasitic or commensal and are related to the ubiquitous human parasite, *Giardia intestinalis*. These parasites are responsible for devastating outbreaks of systemic infections in a wide range of food and ornamental fish and thus represent a significant problem in aquaculture. Despite the detrimental impact of *Spiroucleus* species on an industry of growing importance, they have been little investigated, and information on their metabolism, host-parasite interactions, geographical range, pathogenesis and chemotherapy is scarce. A better understanding of the biology of these parasites is therefore essential for effective disease management. This thesis aimed at advancing the knowledge of *Spiroucleus* parasites, by providing novel information on their growth, metabolism, ultrastructure and treatment. *Spiroucleus vortens*, a parasite of cichlids, was used as a model organism in these investigations. Following optimization of *in vitro* growth conditions, *S. vortens* proved to be a non-fastidious organism, and grew to high densities (2.6×10^6 cells. ml⁻¹) with a very short doubling time (1.79 h), but demonstrated an unusual biphasic pattern of growth. Despite being categorized as anaerobic, the organism exhibited both an unusually high tolerance and affinity for O₂. Garlic and allium-derived thiosulfinates, which are known for their powerful, broad spectrum antimicrobial properties, had a dose-dependant inhibitory effect on *S. vortens*, albeit at doses higher than that required for the inhibition of most microorganisms investigated so far. Metabolic investigations demonstrated that the organism could use glucose, but that the compound was not its preferred substrate. The organism was also found to contain large pools of endogenous substrates, exhibited high proteolytic activity and was capable of rapid phagocytosis of non-soluble particles. Although *Spiroucleus* species were described as lacking hydrogenosomes, *S. vortens* produced H₂ at a very high rate, and ultrastructural and enzymatic studies revealed that despite previous reports, the organism possessed hydrogenosome-like, redox-active organelles. Besides hydrogenosomes, mitosome-like organelles were also detected. Such combination is currently unique in the eukaryotic kingdom and has important implications for current theories of organelles and eukaryotic evolution.

CHAPTER 1: GENERAL INTRODUCTION

Spironucleus are small (typically 10 to 20 μm in length) anaerobic flagellates that belong to the suborder Diplomonadina, family Hexamita, and are related to the ubiquitous human parasite *Giardia intestinalis* (Fig. 1. 1) (Jorgensen and Sterud 2007).

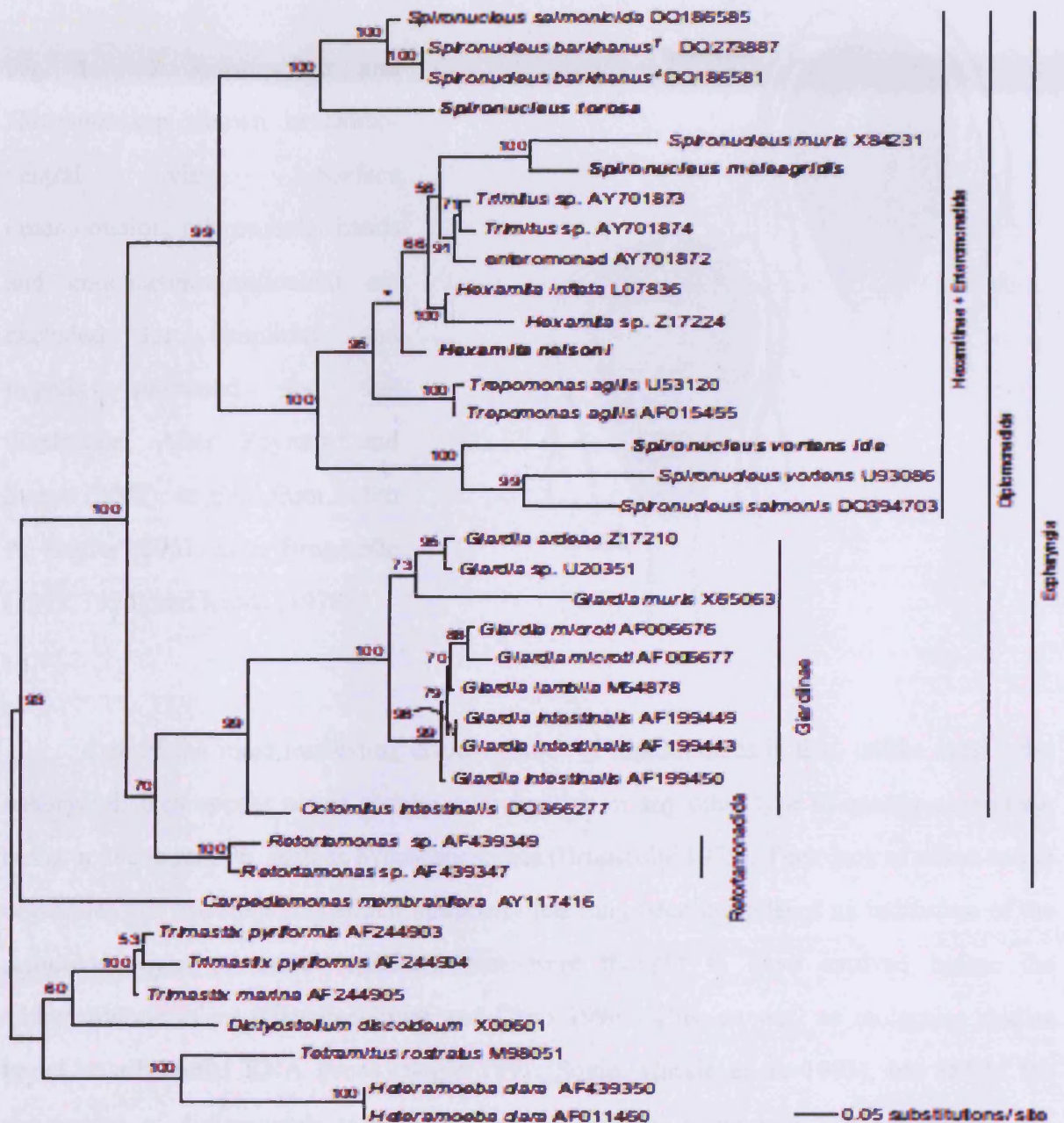
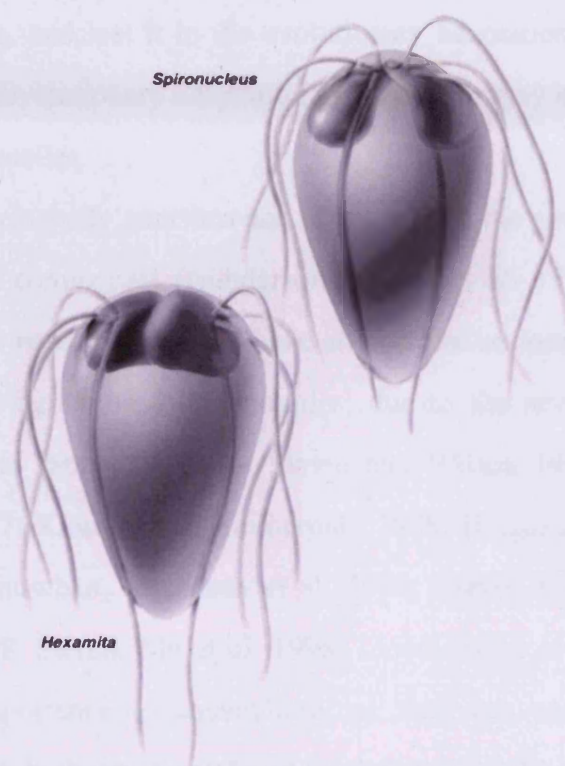


Fig. 1. 1 Maximum likelihood phylogeny of the Eopharyngia based on partial SSU rRNA gene sequence. [†]Isolated from arctic grayling (*Thymallus arcticus*). [‡]Isolated from European grayling (*Thymallus thymallus*). Reproduced from Jorgensen *et al.* (2007).

Characteristically, *Spironucleus* species are pyriform-shaped, with two nuclei and a binary axial symmetry. Six flagella emerge at the front in groups of three and two recurrent flagella run through the body and emerge posteriorly (Fig. 1. 2) (Brugerolle, Joyon et al. 1973; Brugerolle, Kunstyr et al. 1980; Poynton and Sterud 2002; Brugerolle, Silva-Neto et al. 2003).

Fig. 1. 2 *Spironucleus* and *Hexamita* spp. shown in Dorso-ventral view. Surface ornamentation, microtubular bands and endoplasmic reticulum are excluded for simplicity and flagella shortened for this illustration. After Poynton and Sterud (2002), original from Judith A. Stoffer (2001) after Brugerolle (1973, 1974) and Kulda (1978).



One of the most interesting characteristics of diplomonads is that, unlike most other eukaryotes, they appear not to possess mitochondria or any other type of energy-generating, redox active organelle, such as hydrogenosomes (Brugerolle 1975). Their lack of redox-active organelles and apparent subcellular simplicity has long been considered an indication of the primitive origin of these organisms that were thought to have evolved before the endosymbiotic event (Cavalier-Smith and Chao 1996). This, as well as molecular studies based on ribosomal RNA genes (Sogin 1991; Sogin, Hinkle et al. 1993), has led to the assignation of diplomonads to basal eukaryotic lineages along with the Microspora and Parabasalia taxa. The primitive status of these eukaryotes is, however, now increasingly questioned, due identification of several genes of mitochondrial origin (Embley and Hirt 1998; Embley and Martin 1998; Roger, Svard et al. 1998; Roger, Morrison et al. 1999), as

well as small double membrane organelles thought to be vestigial mitochondria, in numerous species of these taxa, including the Microspora, the parabasalia *Trichomonas*, and the diplomonads *Giardia* and *Spironucleus* spp. (Tovar, Fischer et al. 1999; Vivares, Gouy et al. 2002; Williams, Hirt et al. 2002; Bakatselou, Beste et al. 2003; Tovar, Leon-Avila et al. 2003; Regoes, Zourmpanou et al. 2005). This suggests that these organisms may have possessed a mitochondrion or mitochondrion-like structure, and lost it in the evolutionary adaptation to anaerobic lifestyles (Lloyd and Harris 2002). Evolutionary adaptation to parasitism may also have contributed to such secondary loss of organelles.

The diplomonads are indeed almost exclusively parasites and all species in the genus *Spironucleus* appear to be either parasitic or commensal (Gunderson, Hinkle et al. 1995; Cavalier-Smith and Chao 1996). Pathogenic *Spironucleus* species have attracted an enormous amount of attention from the veterinary and agricultural communities, due to the severe pathologies that they cause in companion and farmed animals (Slavin and Wilson 1953; Matthiesen, Kunstyr et al. 1976; Kunstyr 1977; Kunstyr and Ammerpohl 1978; Brugerolle, Kunstyr et al. 1980; Brett and Cox 1982; Januschka, Erlandsen et al. 1988; Harper 1991; Kunstyr, Poppinga et al. 1993; Pennycott 1998; Sterud, Mo et al. 1998; Lloyd, Irvine et al. 2005). These parasites are of particular importance to aquaculture, as they can cause devastating outbreaks of systemic infections in both ornamental and food fish (Nigrelli and Hafter 1947; O'Brien G, Ostland et al. 1993; Sterud 1998b; Sterud, Mo et al. 1998; Tojo and Santamarina 1998; Paull and Matthews 2001; Guo and Woo 2004; Guo and Woo 2004; Jorgensen and Sterud 2006). These ubiquitous flagellates occur in cold, temperate and warm waters, and infect a wide range of freshwater (O'Brien G, Ostland et al. 1993; Tojo and Santamarina 1998; Paull and Matthews 2001) and marine fish (Poynton and Morrison 1990; Sterud 1998a; Sterud, Poppe et al. 2003; Jorgensen and Sterud 2004; Jorgensen and Sterud 2006), as well as shellfish and crustacean (Scheltema 1962; Uzmann and Hayduk 1963; Khouw and McCurdy 1968; Khouw, McCurdy et al. 1968; Schlicht and Mackin 1968; Tojo and Santamarina 1998; Paull and Matthews 2001). Fish farming, an industry of growing importance as wild fish stocks become increasingly depleted, is therefore especially susceptible to outbreaks caused by such diplomonad parasites. For instance, *S. salmonicida*, (formerly *S. barkhanus*, see (Jorgensen and Sterud 2006), has caused several massive

outbreaks of systemic infections in farmed Norwegian Atlantic salmon (Mo, Poppe et al. 1990; Poppe, Mo et al. 1992; Poppe and Mo 1993; Sterud, Mo et al. 1998) and British Columbian Chinook salmon (Kent, Ellis et al. 1992). Gross pathologies include internal haemorrhaging, splenomegaly and granulomatous lesions in spleen and liver (Fig. 1. 3). Transmission is extremely high, especially in the crowded environments of fish farms and mortality in experimental infections approaches 100% (Guo and Woo 2004a&b).

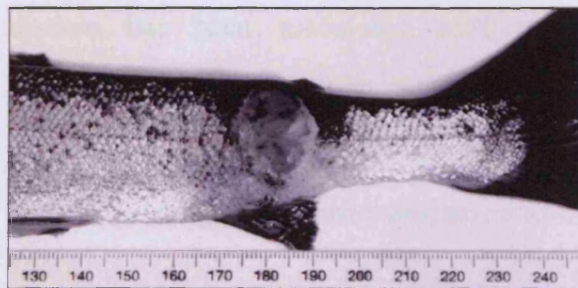


Fig. 1. 3A Skin and muscle ulcerations (top) and exophthalmia (bottom) in Atlantic salmon *Salmo salar* experimentally infected with *Spironucleus barkhanus* (Reproduced from Guo & Woo, 2004b)

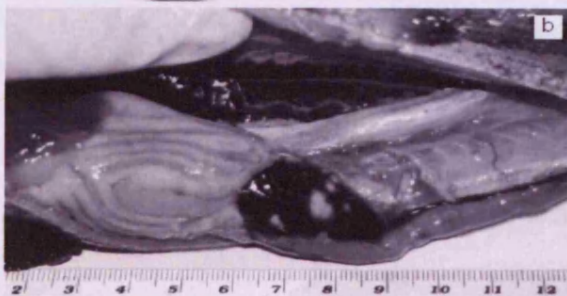
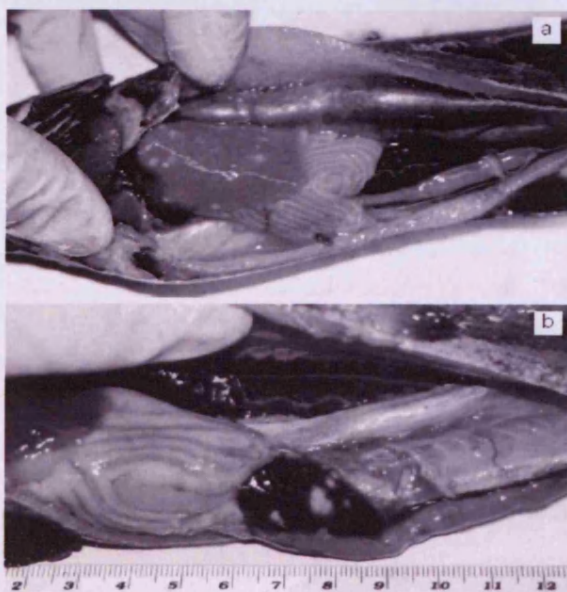


Fig. 1. 3B Splenomegaly and granulomatous lesions in liver (a) and spleen (b) of Atlantic salmon *Salmo salar* experimentally infected with *Spironucleus barkhanus* (Reproduced from Guo & Woo, 2004b).

In the ornamental fish industry, *Spiromucleus* species also cause severe infections in several commercially important species including cichlids (Ferguson and Moccia 1980; Poynton, Fraser et al. 1995; Paull and Matthews 2001) and *S. vortens* has been proposed as the causative agent of hole-in-the-head disease, a common condition generally associated with poor husbandry (Paull and Matthews 2001). The disease is characterised by severe lesions on the head (Fig. 4) and lateral line, as well as internal lesions. In some cases, these lesions are so severe that they literally create a hole in the head of the diseased fish. Hole-in-the-head disease has been associated with systemic infection by *S. vortens*, with parasites systematically recovered in internal organs and skin lesions of diseased fish (Paull and Matthews 2001). It is hypothesised that these intestinal commensals become invasive when the fish is stressed, but exact determinants of the onset of the disease are unknown (Goldstein 1979).

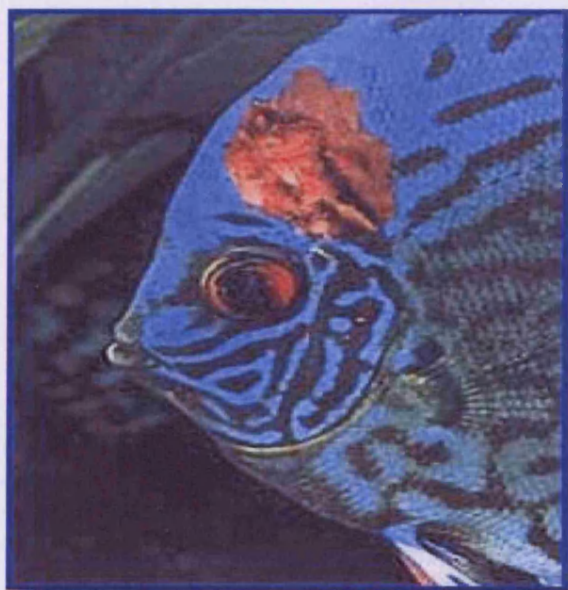


Fig. 1. 5. Discus with a severe case of hole-in-the-head disease. (Reproduced from <http://www.wheelchairanglingandhamradio.co.uk/images/hith-bad.jpg>)

Despite the importance of these parasites, their host range, specificity and mechanisms for transmission are poorly known, as are the geographical range and pathogenesis (Poynton and Sterud 2002; Poynton, Fard et al. 2004). This lack of knowledge is particularly detrimental in disease management, as accurate diagnostic and identification of the source of infection are rendered difficult. Advances in that field are further hindered by the confusion occurring in the nomenclature of hexamitids parasites. Descriptions of the 15 to 20 species

isolated from fish are incomplete and comparisons difficult as few specimens were deposited in reference collections. Besides, numerous isolates have been wrongly identified, sometimes even to the genus level (Poynton and Sterud 2002; Poynton, Fard et al. 2004), due to the use of limited techniques, such as light microscopy (Moore 1922; Davis 1926; Vickerman 1990), within veterinary diagnostic units. Presence of flagellar pockets, location of kinetosomes, shape of nuclei and presence of recurrent axonemes forming a central axis are key characteristics enabling identification of genera (see Table 1. 1), but examination of surface ornamentation and transverse pattern of the flagellar pocket are required for identification at the species level (Poynton and Sterud 2002). This requires specialist tools, such as transmission and scanning electron microscopy, as well as considerable expertise. Although these TEM analysis tools have enabled a more accurate identification of pathogenic isolates to the genus, and even sometimes to the species level (Poppe, Mo et al. 1992; Sterud and Poppe 1997; Poynton and Sterud 2002; Poynton, Fard et al. 2004; Andersson, Sjogren et al. 2007), they remain time-consuming, require expensive, specialist equipment, and can therefore not be employed routinely within veterinary diagnostic units. Moreover, even thorough morphological investigations performed by experts and using modern TEM techniques may fail to identify species accurately, as demonstrated by the recent reassignment of a highly pathogenic isolate of *S. barkhanus* to the new species *S. salmonicida*, on the basis of SSU rDNA sequence analysis (Jorgensen and Sterud 2006). Such molecular-based techniques have enabled to considerably clarify the taxonomy and phylogeny of *Spirotrunculus* parasites (Jorgensen, Alfjorden et al. 2007), however, commercial molecular assays for the routine identification of specific pathogens within diagnostic units, which would prove invaluable in disease management, remain to be developed.

Table 1. 1 Ultrastructural features used to distinguish three of the genera of diplomonad (reproduced from Poynton and Sterud 2002)

Character	<i>Spironucleus</i>	<i>Hexamita</i>	<i>Octomitus</i>
Flagellar pockets (cytostomal canals) ^a	+	+	-
Central axis formed by recurrent axonemes, microtubular bands, endoplasmic reticulum	-	-	+
Two terminal spikes	-	-	+
Shape of nuclei	S-shaped	Spherical	Reniform
Location of kinetosomes relative to nuclei	Sub-apical	External structure	Between
Position of recurrent flagella relative to nuclei	Medial	Lateral	Medial
Supra-nuclear microtubular band	+	+	Reduced
Infra-nuclear microtubular band	+	+	Reduced

^a When present, the recurrent flagella are ensheathed

Metronidazole, a nitroheterocyclic compound selectively toxic to anaerobic protists and bacteria (Edwards and Mathison 1970; Ings, McFadzean et al. 1974; Edwards 1980) is the drug of choice in the treatment of spironucleosis/hexamitidis in the aquarium trade (Tojo and Santamarina 1998; Sangmaneedet and Smith 1999) but is unfortunately of very limited use in coastal aquaculture, where anti-infectious drugs are often the last resort. It is indeed expensive, environmentally hazardous (Richardson and Bowron 1985; Lanzky and Halling-Sorensen 1997) and difficult to administer the adequate quantities of drugs (most often as food pellets) in sea cages or large ponds. Moreover, due to the toxicity of the compound, it has been banned for use in food fish in Europe (L82/14 1998), and no satisfactory treatment for spironucleosis is therefore currently available.

Members of the genus *Spironucleus* are described as anaerobes, however, they are known to infect a wide range of organs and tissues, in which oxygen tensions are likely to vary greatly, and their status of anaerobes may therefore be questionable. Indeed, other diplomonads, such as *G. intestinalis* and *Hexamita inflata* were also initially described as anaerobes, but were subsequently found to benefit from the presence of low concentrations of oxygen, and therefore reclassified as microaerophilic (Ellis, Wingfield et al. 1993; Paget, Kelly et al. 1993; Paget, Manning et al. 1993; Biagini, Suller et al. 1997). Despite lacking cytochromes or respiratory chain components, *H. inflata* was also shown to have a very high affinity for oxygen ($K_m = 15 \mu\text{M}$), (Biagini, Suller et al. 1997; Biagini, Park et al. 2001). Such affinity probably represents a detoxification mechanism and is unlikely to be coupled to energy generation, but enables this organism to survive in environments of fluctuating oxygen tensions, which is of particular interest in a free-living species. While only hypothetical models for O_2 detoxification exist in *H. inflata* (Biagini, Suller et al. 1997; Biagini, Park et al. 2001), O_2 scavenging in *G. intestinalis* has been studied in more depth (Gillin and Reiner 1982; Ellis, Wingfield et al. 1993; Paget, Manning et al. 1993; Brown, Upcroft et al. 1995; Brown, Upcroft et al. 1996). *G. intestinalis* is atypical, in that the conventional enzymes associated with oxidative stress in eukaryotes, namely superoxide dismutase (SOD), catalase and peroxidase are absent (Brown, Upcroft et al. 1995; Brown, Upcroft et al. 1996). Instead, the O_2 detoxification process is similar to that of some bacteria (Stanton, Rosey et al. 1999; Nishiyama, Massey et al. 2001) and occurs through the action of NADH oxidase, whilst superoxide inactivation to H_2O_2 is catalysed by a membrane-bound NADH peroxidase. Oxygen removal in these organisms is therefore dependant on the generation of reduced NAD(P)H cofactors, presumably through glycolysis (see Fig. 1. 6) (Brown, Upcroft et al. 1995; Brown, Upcroft et al. 1996; Brown, Upcroft et al. 1998; Biagini, Park et al. 2001). The preferred O_2 tension for growth of *Spironucleus* species, as well as their tolerance towards the gas and the nature of the O_2 scavenging pathways in these organisms are unknown. Investigation of these matters would be important in understanding the course of infections, as it may explain the affinity of this parasite for particular tissues. It could also contribute to anticipate aggravating environmental conditions for the proliferation of the parasites.

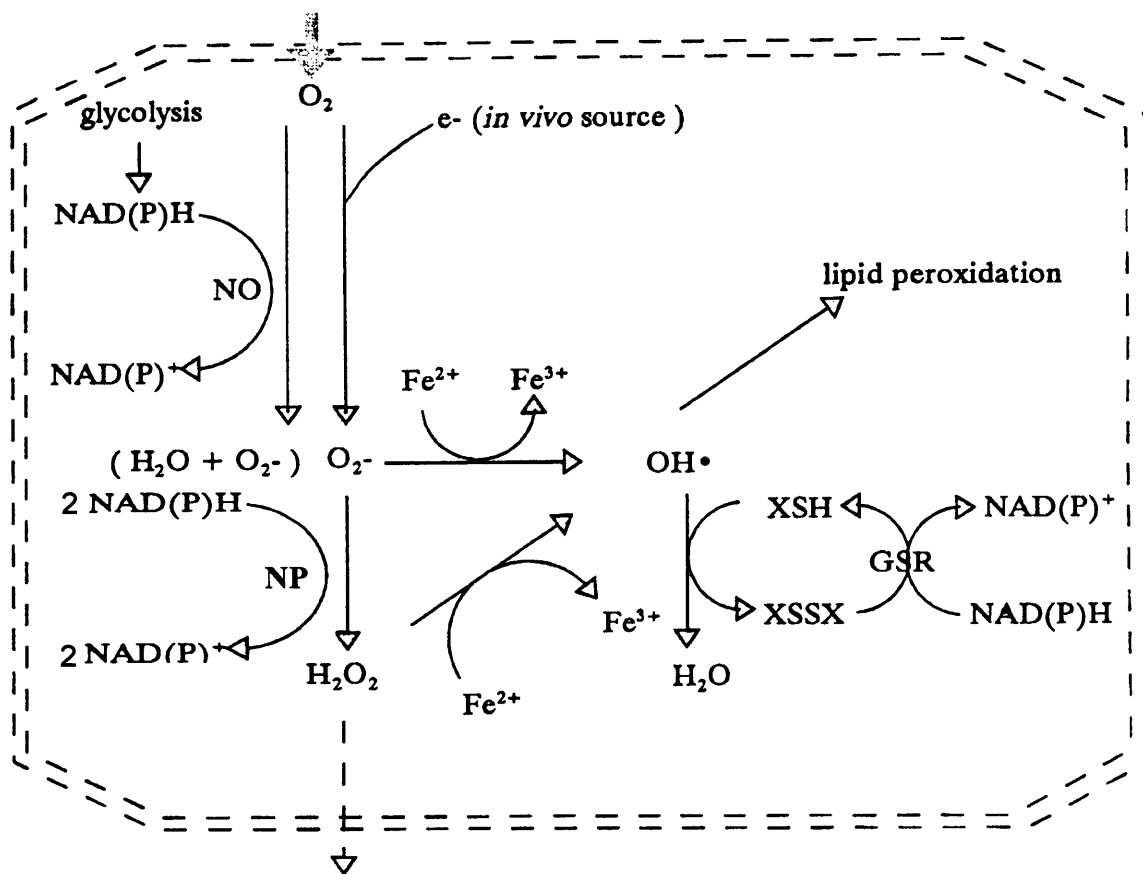


Fig. 1. 6. Proposed mechanism for antioxidant defence in *Giardia intestinalis*. Enzymes involved: NO= NAD(P)H oxidase, NP=NAD(P)H peroxidase. GSR=glutathione reductase

Knowledge of the nutritional and physical requirements for growth of *Spiromucleus* species, as well as understanding of their basic metabolism, would also be invaluable in disease management, however, to date no studies have examined the metabolism of these organisms. The closest studied relatives are *Giardia intestinalis* and the free-living *Hexamita inflata*. Both these organisms lack oxidative phosphorylation but possess an extended glycolytic pathway and derive ATP from substrate level phosphorylation rather than oxidative phosphorylation (Lindmark 1980; Jarroll, Manning et al. 1989; Biagini, McIntyre et al. 1998; Biagini, Yarlett et al. 2003). While the reduced cofactors produced through glycolysis may be reoxidised when the need for O_2 detoxification arises (Fig. 1. 6), replenishment of the oxidised pool of redox cofactors under anaerobic conditions can be problematic. Most anaerobic eukaryotes solve this problem by performing anaerobic respirations, such as alcoholic or

lactic acid fermentations. *G. intestinalis* is atypical, as NAD(P)H regeneration occurs not solely through fermentative pathways, but also by the generation of molecular hydrogen, a bacterial trait, which is excessively rare in the eukaryotic kingdom (Lloyd, Ralphs et al. 2002) (Fig. 1. 7). *G. intestinalis* and *H. inflata* also possess other bacteria-like metabolic traits, such as the arginine dihydrolase pathway (ADH), an amino acid fermentation that produces ornithine from arginine (Schofield, Costello et al. 1990; Schofield, Edwards et al. 1992; Knodler, Schofield et al. 1995; Biagini, McIntyre et al. 1998; Brown, Upcroft et al. 1998; Dimopoulos, Bagnara et al. 2000; Biagini, Yarlett et al. 2003). Such atypical features may represent excellent new targets for antimicrobial drugs. As no satisfactory chemotherapy is currently available for *Spiroucleus* species, determining whether these parasites share such odd, bacterial traits with their diplomonad relatives may therefore greatly also contribute to the development of new approaches in the treatment of spironucleosis.

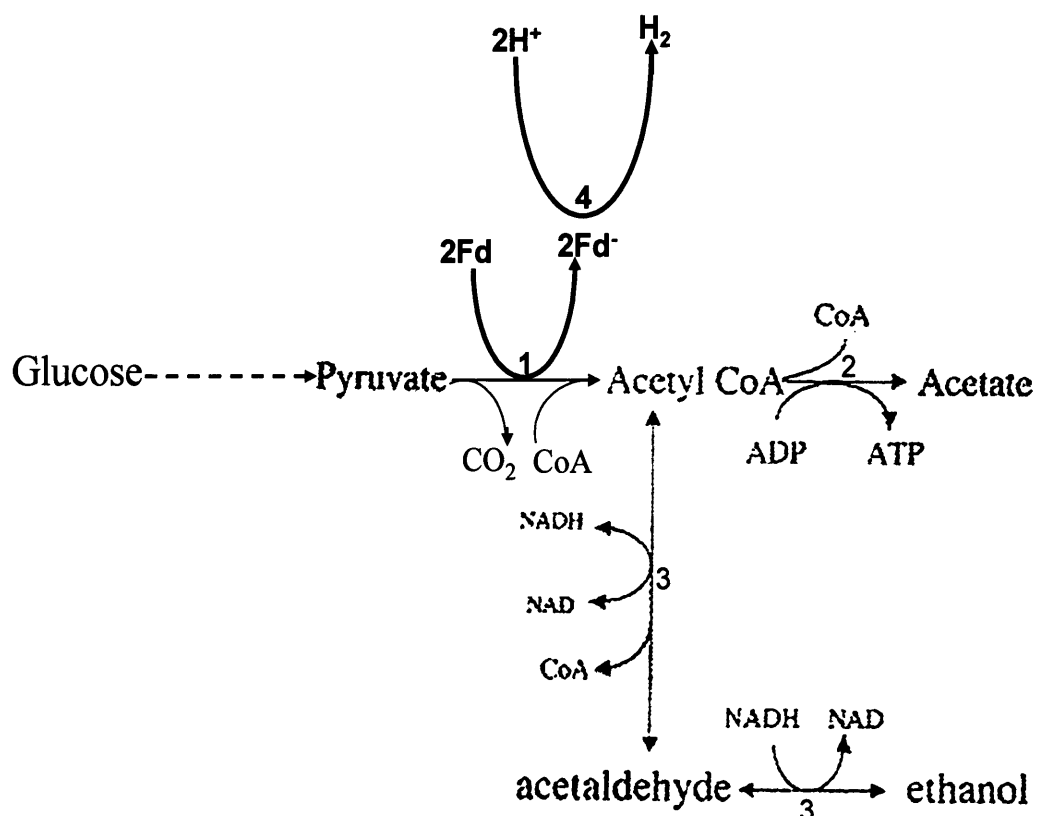


Fig. 1. 7. Proposed pathway for ethanolic fermentation and H₂ production in *Giardia intestinalis*. Enzymes involved: 1. Pyruvate ferredoxin oxidoreductase, 2. acetyl-CoA synthetase, 3. acetate dehydrogenase, 4. FeFe hydrogenase.

AIMS AND THESIS LAYOUT:

Using *Spironucleus vortens* as a model organism, this work aimed at improving our general knowledge of the poorly studied hexamitid parasites. This was done with a view to provide an understanding of the biology of their interactions with the host, as well as develop new treatments. Specific aims were to:

1. Optimize conditions for the *in vitro* cultivation of *S. vortens* and characterize its growth.
2. Define basic nutrient uptake capabilities of the organism by investigating presence of phagocytotic pathways for uptake of non-soluble particles and bacteria.
3. Investigate the presence of atypical metabolic traits described in the related parasite *Giardia intestinalis*, such as H₂ generation and the ADH pathway.
4. Explore basic aspects of *S. vortens* metabolism by :
 - a. Determining the extent of metabolic plasticity by testing metabolic rates and growth over a range of substrates.
 - b. Elucidating basic pathways by identifying products of endogenous and glucose fuelled-metabolism.
5. Investigate *S. vortens* tolerance and affinity towards oxygen.
6. Test the potency of alternative antimicrobials *in vitro*.
7. Scrutinize the details of *S. vortens* ultrastructure, using powerful new tools for cellular imaging.

The work of this thesis is presented into seven self-contained, experimental chapters, which treat points 1 to 7, followed by a short concluding chapter. Chapter 3, 4, 5, 7 & 8 will be prepared and submitted for publication shortly. Preliminary work on the prevalence and host-specificity of *Spironucleus* species, as well as on the development of species-specific molecular markers was also undertaken and results are summarised in Appendices 1 and 2, respectively.

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CHAPTER 2: *IN VITRO* CULTURING AND GROWTH CHARACTERIZATION OF *SPIRONUCLEUS VORTENS*

2. 1 ABSTRACT

Spironucleus vortens is a small, parasitic flagellate, which has been isolated from a diseased angelfish in 1995 and maintained *in vitro* thereafter. Although optimal growth conditions have been described for this organism, we found that success in the establishment of cultures was highly unpredictable. Further investigations revealed that this was due to the high sensitivity of *S.vortens* to the composition of the peptone employed in the culture medium. The organism was found to be otherwise non-fastidious and grew to high densities ($2.6 \times 10^6 \pm 1.13 \times 10^5$ cells. ml⁻¹), with a an extremely short doubling time (1.79 h) in Keister's modified, TY-I-S33 medium. It also proved tolerant of high concentrations of O₂ and resisted extended storage at -180°C in 10% DMSO. Complete axenisation of cultures was challenging and could not be achieved by traditional means of antibiotic treatment alone. Instead, it required physical segregation of trophozoites from bacteria, a process based on the differential motility of both types of microorganisms. Analyses of growth curves generated by automated optical density monitoring (Bioscreen C) suggest that nutrient concentration in culture medium exceeds that needed by the organism for optimal growth. Interestingly, growth monitoring also revealed that the organism displays an atypical, biphasic growth curve, in which the two distinct log phases are not separated by a visible lag phase and the second exponential growth rate is significantly faster than the first. Such peculiar growth pattern may result from a metabolic switch, following the exhaustion of one substrate or from the conditioning of the culture medium and removal of an inhibitory substance. Further studies are required to address this question.

2. 2 INTRODUCTION

In vitro culture offers a reliable and convenient source of cells, providing high densities of trophozoites free of host material that can be easily harvested for taxonomical studies, biochemical investigations or infection trials. Parasites are notoriously fastidious to maintain *in vitro*, and optimisation of *in vitro* culturing conditions is fundamental to studying the organisms (Puentes, Diaz et al. 2000; Bixler, Lerner et al. 2001; Gupta, Goyal et al. 2001; Spiliotis, Tappe et al. 2004; Correa, Santos et al. 2005; Porter-Kelley, Dinglasan et al. 2006; Brehm and Spiliotis 2008; Dias Costa, Soares et al. 2009; Spiliotis and Brehm 2009). Knowledge of optimal culturing conditions also provides valuable insights into nutritional and physical requirements, which is directly relevant to understanding pathogenesis, host specificity and transmission (for example, which conditions favour colonisation and proliferation of parasites within the host or their survival outside the host).

The first *in vitro* culture of *Hexamita salmonis* was established by Uzmann and Hayduk (1963) but to date, only four hexamitid fish parasites are routinely cultured: *H. salmonis*, (ATCC 50329) (Buchmann and Uldal 1996), *Spirotrunculus vortens* (ATCC 50386) (Poynton, Fraser et al. 1995; Sangmaneeet and Smith 2000), *S. barkhanus* (ATCC 50380) (Sterud 1998b) and an undescribed diplomonad isolated from Chinook salmon (ATCC 50330) (Kent, Ellis et al. 1992). *S. torosa*, an exclusively marine species, still eludes *in vitro* culturing (Poynton and Morrison 1990; Sterud 1998a). Defined temperature ranges for optimal growth of three of the four species that are currently successfully maintained *in vitro* (*S. vortens*, *S. barkhanus* and *H. salmonis*) are available (Buchmann and Uldal 1996; Sterud 1998; Sangmaneeet and Smith 2000; Paull and Matthews 2001; Sterud and Poynton 2002; Sterud, Poppe et al. 2003), but reports on nutritional requirements are conflicting (see Table 2. 1). For instance, growth of *S. vortens* isolates may be supported by a bile-supplemented, Keister's modified TYI-S-33 medium alone or require addition of fresh fish liver. Different species also seem to have very different requirements; as exemplified by the observation that *S. barkhanus* isolated from grayling gall bladders or Atlantic salmon abscesses reportedly grows in Keister's medium, while *Spirotrunculus* isolates from rainbow trout intestine require the addition of minimal essential medium (Buchmann and Uldal 1996; Sterud 1998b;

Sangmaneedet and Smith 2000; Paull and Matthews 2001; Sterud and Poynton 2002; Sterud, Poppe et al. 2003).

Table 2. 1. Culturing conditions reported for the different isolates of *Spironucleus* species currently maintained in vitro.

Species	Source	Culture medium	Temp. (°C)	Reference
<i>Spironucleus vortens</i>	Lip lesion, angelfish, Florida (ATCC 50386)	Keister's modified TY-I-S33	25 [22-34]	Sangmanee det & Smith 2000
	Intestine, internal organs and head lesions of cichlids with hole in the head disease, UK	Keister's modified TY-I-S33 supplemented with fresh <i>Oreochromis niloticus</i> liver	25	Paull & Matthews 2001
	Intestine of wild Ide, river Leira, Norway	Keister's modified, TY-I-S33	15 [5-15]	Sterud & Poynton 2002
<i>Spironucleus barkhanus</i>	Gall bladder of wild grayling, Norway	Keister's modified TY-I-S33	15 [5-20]	Sterud 1998
	Muscle abscess of farmed Atlantic salmon, Norway	Keister's modified TY-I-S33	10 [5-20]	Sterud 1998
	Blood, liver and kidney of farmed Atlantic char, Norway	Growth not supported by Keister's modified TY-I-S33	NA	Sterud et al. 2003
<i>Spironucleus salmonis</i> (previously <i>Hexamita salmonis</i>)	Intestine of wild rainbow trouts, Denmark	MEM with 10% bovine serum. Not supported by Keister's modified TY-I-S33	10 [5-10]	Buchmann & Uldal 1996

Some of these discrepancies may be explained by the variations between isolates, but may also be linked to the variation in the amino acid or salt composition of complex media components, such as casein peptones, yeast extract or sera. Several authors have reported

failure of cultures after fresh medium was made or the origin of peptone changed (Sterud personal communication) and other precise studies highlight the uncertainties associated with the variations in composition of complex media and their sometimes profound influence on growth rates, yields, as well as the immunological and morphological characteristics of microorganisms (Gray, O'Reilly et al. 2006; Gray, Muller et al. 2008) It is thus possible that parasites are sensitive to fine changes in media composition, and achievement of high density cultures may require optimization of culture conditions for every new isolate in each laboratory.

Elaboration of a nutritionally appropriate culture medium for parasites is challenging, but another difficulty in the establishment of *in vitro* cultures, is to successfully remove contamination by other microorganisms, such as bacteria. This can prove difficult, as few antibiotics are truly bacteriocidal in liquid cultures, and to avoid resurgence of the contaminant, they must be added to culture on a permanent basis. The use of such continuous antibiotic pressure not only eventually selects for antibiotic resistance, but is also likely alter the normal metabolism and cause damage to the parasite (Kemp, Sherr et al. 1993; Pallasch 2003). For ultrastructural or microscopic studies, the perfect axenisation of cultures is not essential. It is also true of some types of short-term biochemical experiments, in which the impact of the presence of a low number of contaminants, may be negligible. However, during longer term analyses involving live cells, bacterial numbers, even if very low to start with, can increase exponentially as the antibiotic pressure is removed, and therefore significantly alter results. Whenever possible, perfect axenisation, without continuous antibiotic usage is therefore desirable.

In this study, *S. vortens* cultures were axenised, culturing conditions optimized, and growth of the parasite under these conditions was described by cell counting and automated optical density monitoring.

2. 3 MATERIALS AND METHODS

Optimisation of growth conditions. *Spironucleus vortens*, ATCC 50386, was obtained from Prof. J. Kulda. In order to optimize growth, several modifications in the composition of culture medium, subculturing protocol and growth temperature were attempted. In the first treatment, trophozoites were cultured at 20°C in 15 ml screw-capped Falcon tubes on Keister's modified TYI-S-33 medium, without vitamin, and containing (per litre): pancreatic digest of casein (Oxoid), 20 g; yeast extract (Oxoid), 10 g; glucose (Sigma), 10 g; bovine bile (local slaughter house), 1ml; NaCl (Merck), 2 g; L-cystein. HCl (Sigma), 2 g; ascorbic acid sodium salt (Fluka), 0.2 g; K₂HPO₄ (Merck), 1 g; KH₂PO₄ (Merck), 0.6 g; ferric ammonium citrate (Sigma), 22.8 mg, heat-inactivated newborn calf serum (Gibco). The pH was adjusted to 6.8 with NaOH prior to filter sterilization (0.22 µm pore size). Subculturing was performed routinely at 72 h intervals, and phase-contrast microscopy was used to monitor the viability of cultures, based on a visual estimate of density and motility.

Growth was then similarly monitored at 20°C, in Keister's modified culture medium, but and using a peptone from a different origin (casein digest peptone, BBL), with (treatment 2) or without (treatment 3) addition of a solution of vitamins, at 30 ml .L⁻¹ obtained by mixing aseptically the following: solution 1, 0.4 ml; solution 2, 1.2 ml; solution 3, 0.4 ml; solution 4, 100ml, distilled water; 18 ml; with solution 1 containing, D, L-6,8-Thioctic acid, oxidized (Sigma), 100 mg; absolute ethanol, 100ml; solution 2 containing, vitamin B12 (Sigma), 40 mg; distilled water, 100 ml; solution 3 containing, tween 80 (Sigma), 50g; absolute ethanol, 100 ml; and solution 4 containing, per litre, α Tocopherol phosphate, disodium salt, 0.025 mg.; d-biotin, 0.025 mg; calciferol (vitamin D2), 0.250 mg; calsium D-(+)-pantothenate, 0.025; choline chloride, 1.250 mg; folic acid, 0.025 mg; i-inositol, 0.125 mg; menadione, 0.025 mg; niacin, 0.0625 mg; p-aminobenzoic acid, 0.125 mg; pyridoxal-HCl, 0.0625 mg; pyridoxine, HCl, 0.0625; Riboflavin, 0.025; thiamine HCl, 0.025 mg; vitamin A, 0.250 mg; vitamin B12, 10 000 mg.

Growth was also attempted in Keister's modified medium without vitamin mix, using casein peptone (BBL) in 15 ml screw-capped Falcon tubes, with (treatment 4) or without (treatment 5) a 6 ml headspace, and monitored by light microscopy, as described above. Survival of

trophozoites in 5ml of buffer, within a 50 ml beaker, was monitored by light microscopy at hourly intervals, for 10 h.

In a 6th treatment, trophozoites were subcultured in the Keister's modified culture medium, without vitamin mix, using casein peptone (BBL), in Falcon tubes with a 6 ml headspace, but replacing heat-inactivated newborn calf serum (Gibco) with heat-inactivated foetal calf serum (Gibco).

Optimisation of conditions for cryopreservation. Cryopreservation of log phased cultures (approximately 10^6 cells. ml⁻¹, as determined using an improved Neubauer haemocytometer, see below) was attempted in liquid nitrogen using 5% DMSO or 5% glycerol as cryopreservants. Culture samples were mixed aseptically with the cryoprotectant then cooled overnight at a controlled rate (about 1°C per min) in a bath of isobutanol placed in a -80°C freezer. After 48 h, 2 months and 1 year, revival was attempted for the DMSO and glycerol maintained cultures. Cryo vials were thawed at ambient temperature without shaking and 1 ml was added to 9 ml of fresh culture medium.

Axenisation of cultures. *Spironucleus vortens*, ATCC 50386, was obtained from Prof. J. Kulda (Charles University, Prague, Czech Republic). It was first isolated from a lip lesion in a freshwater angelfish in Florida in 1991 (Poynton et al. 1995). Microscopic examination showed that cultures were contaminated with low numbers of a gram negative, rod-shaped bacteria. New cultures ordered from the American type culture collection (ATCC, culture reference 50386), were found to also contain a Gram-negative, rod shape contaminant. This contaminant was plated on blood and TYM agar, under aerobic conditions or in an anaerobic jar, and incubated at 20°C for 96h. A mixture of 1000 units of Penicillin G, 250 µg. ml⁻¹ amikacin, 100 µg. ml⁻¹ streptomycin and 100 µg. ml⁻¹ colistin sulphate were added to the *S. vortens* cultures during 3 consecutive subcultures (over 72 h). Cultures were grown on plates at 20°C, in the absence of antibiotics, under aerobic or anaerobic conditions, on TYM or blood agar, 48 h after the start of the antibiotic treatment. They were then similarly plated after the last antibiotic passage, 48 h after cultures were transferred to fresh, antibiotic-free culture medium. Finally, cultures were plated 3 subcultures after the last antibiotic passage. In

order to avoid any external contamination, cultures were handled in a Class II, laminar overflow cabinet, in which filters were regularly replaced. Prior to opening the cultures, the hood was wiped with 70% ethanol and UV-sterilized for 20 min. All pipettes and tips were autoclaved at 120°C for 30 min, dried thoroughly and UV-irradiated for 20 min. Hands and arms were scrubbed with antimicrobial soap (), and scrubbed in 70% ethanol for 2 min prior to handling the cultures.

In order to separate bacteria from motile trophozoites, five soda glass Pasteur pipettes were bent into a S-shape in a butane torch, autoclaved for 20 min at 120°C, and filled with 4ml of sterile, Keister's modified TY-I-S33 culture medium, containing 0.5% of freshly autoclaved, low melting point agarose. The distal ends of the pipettes were then sealed by melting the glass in the butane-fuelled flame. Approximately 10^6 log phased trophozoites were passaged three times in antibiotics, immediately harvested by centrifugation at 800 g for 4 min at 5°C in a Beckman Coulter Avanti J-E Centrifuge (Fullerton, California, USA), washed twice in PBS, and inoculated at the top end of the pipettes. After 10 h, and at 2 h intervals, the distal end of a pipette was carefully cracked, using sterile forceps, and the first drop of culture medium was recovered aseptically and inoculated into fresh culture medium. The second drop was recovered onto a clean glass slide, and presence of trophozoites was investigated by light microscopy. Resulting cultures were then tested for the presence of bacteria by microscopy and plating on blood and TYM agar under aerobic and anaerobic conditions.

Monitoring of cell numbers during growth in Keister's modified medium using an Improved Neubauer haemocytometer (Weber Scientific International, UK). Duplicate falcon tubes were filled with 10 ml of Keister's modified TY-I-S33 medium, inoculated with 300 µl of log-phased cultures and incubated at 25°C for 120 h. Small volumes (200 µl) were removed aseptically from tubes after vortex homogenisation after 24, 48, 96 and 120 h incubation, and mixed with 200 µl of 4% (w/w) solution of paraformaldehyde. Samples were diluted in order to contain approximately 200 to 1000 cells. μl^{-1} , thoroughly homogenised using a vortex mixer, and carefully loaded in the chamber of an improved Neubauer haemocytometer, as recommended by the manufacturer. The number of cells in present in the central square of the chamber (0.1 µl) was then counted in 4 replicates for each sample.

Monitoring of cell density during growth in Keister's modified medium in Bioscreen C.

Bioscreen C (LabSystems, Finland), an automated optical density-monitoring system, was used to monitor growth of *S. vortens* in 100-well honeycomb plates. Each well filled with 340 µl of full strength, or diluted (final concentration 1:3) Keister's modified TY-I-S33 culture medium was inoculated with 10 µl of log-phased cultures and turbidity was measured by OD on a vertical light path, using a wideband filter (420-580 nm) every 20 min for 120 h. Heat transfer fluid circulation maintained a constant temperature of 25°C and plates were shaken at low amplitude for 5 sec before each reading. OD measurements were logged, plotted against time, and exponential growth rates (μ), doubling times (T_d) and final yields were extrapolated from growth curves by applying the following formula:

$$\mu = 2.303(\log_{10}OD_{t_2} - \log_{10}OD_{t_1}) / (t_2 - t_1)$$

$$T_d = \ln 2 / \mu$$

$$\text{yield} = OD_{t_f} - OD_{t_0}$$

In order to determine whether medium dilution had an effect on exponential growth rates or final growth yield, Student T-tests were performed using Minitab 13 at a 95% confidence level.

2. 4 RESULTS

2.1 Optimisation of growth conditions. In the culture medium containing Oxoid's digest of casein, cultures failed to grow, and no live trophozoites could be found 96 h after the inoculation of the culture medium with 200 µl of live trophozoites. When the Oxoid peptone was replaced by a BBL peptone, trophozoites grew to high densities ($2.07 \times 10^6 \pm 32500$ cells. ml^{-1} , N=8) after 48 h of incubation in Keister's modified culture medium. Addition of the vitamin mix did not seem to significantly increase cell density after 48 h of growth. Growth of trophozoites in culture medium was not impaired by the presence of a large headspace of air in the culture tube and the organism remained actively swimming after 10 h of incubation in unstirred buffer at 20°C, in a beaker with a large headspace. Substitution of newborn calf serum with foetal calf serum also did not appear to affect growth of the organism. Trophozoites were thereafter cultured at 20°C, in Keister's modified TY-I-S33 culture

medium, with BBL casein peptone, newborn calf serum, without vitamin mix, and with a 6 ml headspace.

2. 2 Optimisation of conditions of cryopreservation. Growth of *S. vortens* was achieved from stocks preserved for 48 h in 10% DMSO, but not from cultures preserved in glycerol. DMSO preserved cultures were successfully recovered after 2 and 12 months of cryopreservation in DMSO.

2. 3 Axenisation of cultures. Under aerobic conditions, plating of 2 ml of *S. vortens* cultures on blood or TYM agar, resulted in the growth of a continuous layer of a single, Gram-negative, rod-shaped bacteria after 48 h of incubation. Rods were approximately $0.2 \times 3 \mu\text{m}$ and formed round, white colonies. Approximately 5.12×10^3 cfu were present in log-phased cultures. In anaerobic jars, no growth was observed after 96 h incubation at 25°C . During antibiotic treatment, no bacteria were observed in cultures and plating on blood and TYM agar yielded no growth of bacteria. In one subculture, after cessation of antibiotic treatment, low levels (50 cfu. ml^{-1}) of a rod-shaped, Gram-negative bacterium were present in log-phase cultures, and after 3 subsequent subcultures, their number increased to approximately $1.07 \times 10^4 \text{ cfu. ml}^{-1}$.

Trophozoites reached the distal end of the S-shaped pipette 12 h after inoculation. Plating of cultures 48 h after recovery from the distal end of the cultures revealed no bacteria, this was still true in all subsequent subcultures.

2.4 Growth curves

Growth curves of *S. vortens* obtained in full strength and 1:3 diluted culture medium at 25°C by haemocytometer and automated optical density monitoring (Bioscreen) are presented in Figure 2. 1. Haemocytometer-based cell counting showed that *S. vortens* demonstrated a logarithmic growth with a short lag phase, and that maximum yield of cultures after 60 h was $2.60 \times 10^6 \pm 112500 \text{ cells. ml}^{-1}$, (N=8). Optical density monitoring provides more accurate information, due to the availability of more data points. Triplicate curves show that the organism exhibits a biphasic growth, where the initial exponential growth rate (μ) during a

first log phase is followed by a faster growth rate (μ_2), during a second log-phase. Maximum cell density is reached after approximately 60 h of growth. As expected, final yield and exponential growth rates, as well as cumulated duration of both log phase were higher in full strength medium than in diluted culture medium, but these differences were not proportional to the dilution factor. Respective length of individual log phases also varied greatly according to the concentration of the culture medium. Results are summarised in Table 2. 2.

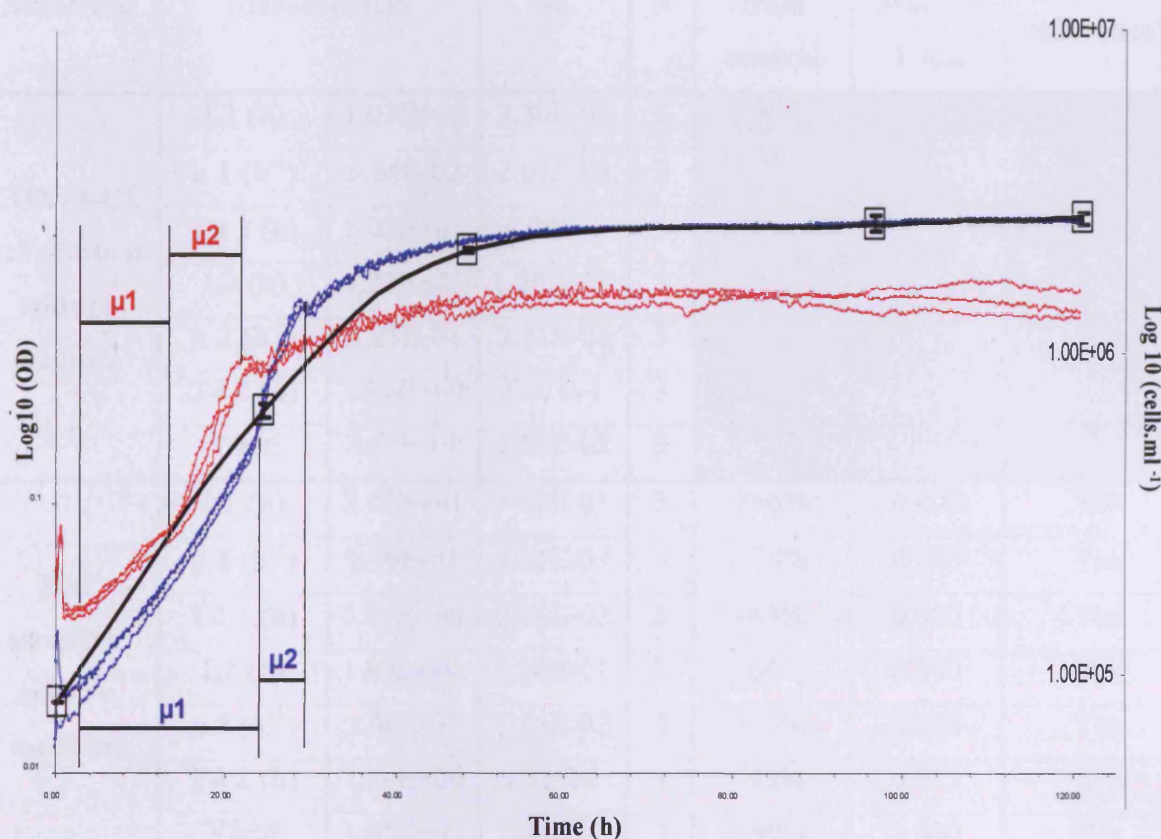


Figure 2. 1. Growth curves of *Spironucleus vortens* in full-strength and 1:3 diluted TY-I-S33 culture medium. Optical density of *S. vortens* cultures in full strength (blue plot) and 1:3 diluted (red plot) culture medium, was recorded in triplicate every 20 min, over 120 h by automated optical density monitoring (Bioscreen C). Cell numbers in full strength culture medium were counted at 0, 24, 48, 96 and 120 h using an improved Neubauer haemocytometer and are plotted in black. Values represent the mean of 8 replicates and standard errors are plotted around the means.

Table 2. 2. Growth parameters of *Spironucleus vortens* in full strength and 1:3 diluted culture medium. Length of the first (L1) and second (L2) log phase, exponential growth rate during the first and second growth rate (μ_1 and μ_2 , respectively), doubling time during the first and second log phase (Td1 and Td2, respectively), and total yields are derived from automated optical density monitoring of *S. vortens* solutions obtained using Bioscreen C.

Substrate	Measurement		SE	N	Variation from control	P-value Student T test	Significant difference?
CONTROL 1:3 dilution culture medium	L1 (h)	1.03E+01	3.30E-01	3			
	μ 1 (h ⁻¹)	6.84E-02	2.67E-03	3			
	Td 1 (h)	1.02E+01	4.09E-1	3			
	L2 (h)	5.23E+00	1.20E+00	3			
	μ 2 (h ⁻¹)	2.25E-01	2.31E-02	3			
	Td 2 (h)	3.14E+00	5.21E-1	3			
	Yield	5.55E-01	1.50E-02	3			
Full strength culture medium	L1 (h)	2.02E+01	1.10E-01	3	+96%	0.000	Yes
	μ 1 (h ⁻¹)	1.19E-01	1.41E-03	3	+74%	0.000	Yes
	Td 1 (h)	5.81E+00	6.91E-02	3	-43%	0.000	Yes
	L2 (h)	1.89E+00	2.90E-01	3	-64%	0.000	Yes
	μ 2 (h ⁻¹)	3.90E-01	2.15E-02	3	+75%	0.006	Yes
	Td 2 (h)	1.79E+00	1.16E-1	3	-43%	0.013	Yes
	Yield	1.06E+00	3.06E-03	3	+90%	0.000	Yes

2. 5 DISCUSSION

This study shows that *Spironucleus vortens* is highly sensitive to variations in the composition of peptone used in the culture medium. Peptones are complex animal products, and their amino acid composition has been shown to vary extensively depending on the manufacturer, the batch or the length of storage, even under the recommended conditions of temperature and humidity. Studies on *Salmonella* species thus have shown that such variations can have a profound influence in the growth and morphology of microorganisms (Gray, O'Reilly et al. 2006; Gray, Muller et al. 2008). In the absence of a chemically defined culture medium, this variability in peptone composition forces investigators to buy products in bulk, in order to ensure continuity of cultures and consistency in their metabolic processes. Strangely, although *S. vortens* appears extremely sensitive to variations in the composition of peptones, the origin of the serum added to the culture medium did not seem to influence growth, as trophozoites grew equally well with foetal or newborn calf serum. *S. vortens* also seemed capable of synthesising or scavenging vitamins from the growth medium, as it did not require addition of the vitamin mix.

Unlike strict anaerobes, which cannot grow if any headspace of air remains in the tube, *S. vortens* grew in high density cultures in tubes with a large headspace, suggesting a high tolerance for oxygen. This is confirmed by the observation that trophozoites not only survive but continue swimming, even after 10 h in unstirred buffer with a large headspace of air, and in the absence of any reducing agent.

Great care was taken to avoid contamination of cultures; however, low levels of contamination were found in cultures grown from frozen stock provided by the ATCC, suggesting that ATCC cultures were probably not axenical. Kulda and colleagues, who have successfully maintained the same ATCC culture for several decades, routinely add high concentrations of amikacin and penicillin to the growth medium of *S. vortens*, also suggesting that axenisation of the standard ATCC stock culture was not perfect. While such low levels of contamination would not be problematic in morphological investigations, short term experiments involving live cells, or for some assays performed on cell-free extracts, they can cause significant variations in the results of long-term mass spectrometer, bioscreen or NMR

experiments involving live cells. Indeed, as soon as the antibiotic pressure is removed, bacterial numbers can increase exponentially, and at a much faster rate than that of the more complex, eukaryotic parasite. The stress generated by the centrifugations and transfer steps from culture medium into buffer required in such experiments would also be likely to favour bacterial growth over that of the more sensitive eukaryotic cells. As this PhD focussed on long term experiments involving live cells, complete axenisation had to be attempted. The current study showed that the contaminant present in the ATCC cultures could not be completely eliminated by traditional methods, as even the use of very aggressive antimicrobial treatments failed to permanently remove the contaminant. This is probably due to the well-documented ability of bacteria to lay dormant, or even form highly resistant phenotypes or spores, which enables them to survive during antibiotic treatment (Graham and Bott 1975; Russell 1999; Lee, Costerton et al. 2007). In fact, it appears that the use of antibiotics ultimately increased contamination levels, as high concentrations of such compounds probably caused some damage to the parasites, which may have subsequently been less capable of competing with the resurging bacteria when antibiotics were removed. Physical separation of parasites from the contaminant, based on differential motility was more successful, as it appeared to remove contamination on a more permanent basis. It should be noted that the flagella-based motility of *S. vortens* is quite extraordinary powerful, as the organism was able to swim through 0.5% agar in less than 12 h. Such incredible motility almost certainly contributes to the invasiveness and virulence of this remarkable organism.

Growth of *S. vortens* in Keister's modified medium is logarithmic, which is typical of microorganisms that divide by binary fission and cultures reached very high densities (2.60×10^6 cells. ml⁻¹ in 120 h.). Flagellated parasitic eukaryotes, such as *G. intestinalis* and *T. vaginalis*, are known to have unusually fast doubling times, which can be lower than 9 h for *G. intestinalis* (Boreham, Phillips et al. 1984) and 2.3 h for *T. vaginalis* (Paget and Lloyd 1990). However, in *S. vortens*, doubling time in the second log phase is 1.79 h (5.81 h in the first log phase), which is extremely short for a eukaryotic cell. Such potential for rapid multiplication, is quite extraordinary and may also contribute to the invasiveness of this organism.

While one might expect the total yield in full strength culture medium to be 3 times higher than in 1:3 diluted culture medium, total yield of *S. vortens* in full-strength culture medium is only 1.91 times higher than that obtained in 1:3 diluted culture medium, which suggests that the medium contains much more nutrients than can be used by the organism before growth becomes inhibited by the accumulation of waste products. This corroborates results obtained by amino acid analysis (see Chapter 5), which show that after 4 days of growth, a large excess of free amino acids still remains in the culture medium. *S. vortens* has a very short lag phase, which is not surprising as they are subcultured from an identical culture medium, and therefore the need for metabolic adjustments and synthesis of new enzymes prior to continuing growth must be minimal.

The biphasic growth curves exhibited by *S. vortens* are quite unusual. Indeed, on the first log phase, the exponential growth rate is quite low, while on the second log phase, it increases sharply. This type of curve is sometimes observed when an organism runs out of its substrate of choice and starts using another. However, as new enzymes usually have to be upregulated to use the new substrate, a short period of latency tends to precede the second log-phase (Batzing and Claus 1971; Haarasilta and Oura 1975; Pyun, Modak et al. 1989; Shadowen and Sciortino 1989; Lewis, Northcott et al. 1993; Meunier and Choder 1999). This is not the case in *Spironucleus*, where the second log-phase follows the first without any pause or even decline of growth rate. This could indicate that all enzymes for exploitation of these new substrates are already available in sufficient concentrations within the cell. While unusual, this is possible, as in order to store some metal ions, such as iron, which is toxic while unbound, microbial cells are known to sometimes use siderophores, as storage proteins (Matzanke, Bill et al. 1987) thus accumulating large stores of them. Similar accumulation of functional enzymes could in turn greatly reduce the amount of time necessary to adapt to a new substrate. Large accumulations of ferredoxin were detected at the anterior end of trophozoites by immunoconfocal microscopy (see Chapter 8), thus rendering this hypothesis all the more likely.

Interestingly, a 1:3 dilution of the culture medium induces changes, not just in the final yield, but also in the relative length of both log phases. In diluted culture medium, the first log phase is much shorter than in full strength culture medium, and the ratio of $L1 \times \mu l$ in full

strength and diluted culture medium is 3:41. While this could hardly be deemed surprising, as cells would be expected to run out of a specific nutrient approximately 3 times faster in 1:3 diluted culture medium than in full strength culture medium, the variations in length and $L \times \mu$ (=yield) observed in the second log-phase are more puzzling.

Indeed, the yield during the second log-phase ($L_2 \times \mu_2$) in the diluted culture medium (1.20) is much higher than in full strength culture medium (0.7). If the presence of two distinct log phases is indeed caused by a switch in nutrient source, the same 1:3 ratio found in the yields of the first log phase ($L_1 \times \mu_1$) would also be expected in the second log-phase. The most simple explanation for this apparent abnormality is that, in full strength medium, the second log phase is reached at a higher cell density than in diluted culture medium. The concentration of accumulated toxins would therefore be higher, and start impairing growth sooner, thus shortening the length and yield of the second growth phase. Another explanation would be that the metabolism in the second log phase is not based upon a nutrient provided in the culture medium, but on endogenous metabolism or on a compound that was excreted in the first log phase. However, neither of these options seem likely, as the first would entail that $L_2 \times \mu_2$ be proportional to $L_1 \times \mu_1$ and the second that $L_2 \times \mu_2$ be higher in full strength than in diluted culture medium. Indeed, while it is not unknown for organisms, such as *Saccharomyces cerevisiae* to start using a metabolite accumulated during growth (thus exhibiting biphasic growth curves) (Haarasilta and Oura 1975; Schaarschmidt and Lamprecht 1977; Lewis, Northcott et al. 1993; Ohlmeier, Kastaniotis et al. 2004), growth yield in the second part of such growth would be directly proportional to the rate of accumulation of the metabolite during the first phase of growth. Moreover, if *S. vortens* was shown to possess large stores of endogenous metabolites, the availability of such compounds ought to be proportional to medium concentration. However unlikely, this hypothesis cannot be discarded, as many complex factors and chemical interactions could contribute to the seemingly inverse relationship between yields in the two log phases.

An alternative explanation for the biphasic growth of *S. vortens*, could be the presence of an inhibitory compound in the culture medium, one that would be removed or inactivated, not gradually through metabolism, but indirectly through the conditioning of the culture medium. For instance, changes in pH or quorum-sensing-controlled release of proteases could

cause irreversible denaturation of an inhibitor protein, thus leading to a sudden increase in growth rate. Some of the ingredients of the culture medium, such as bile, have been found to slow the growth of *S. vortens* (Sangmaneeet and Smith 2000), presumably due to membrane disruption resulting from the detergent properties of some bile components. It is therefore possible that culture medium could contain some inhibitory substance, however it is difficult to speculate on the identity of such a potential inhibitor. Oxygen, another of the most obvious candidates, is unlikely to be responsible, as the onset of the second log phase occurs much earlier in diluted culture medium than in full-strength culture medium. As oxygen concentrations would not be lower in diluted culture medium, but may in fact be slightly higher (due to the lower concentration of reducing agents), the earlier onset of the second log-phase in diluted medium is inconsistent with the theory of oxygen inhibition.

Further investigations are clearly required to investigate the various hypotheses regarding the biochemical basis of the atypical, biphasic growth curve of this organism. Nevertheless, this study demonstrates that *Spironucleus vortens* is highly sensitive to changes in peptone composition, but that provided that the right peptone is used, it can be readily grown to high densities, without additional supply of complex and costly vitamin mixtures. Moreover, it has an extremely low doubling time and is sufficiently tolerant of oxygen that special measures to avoid exposure of cultures to air need not be taken, thus making this organism non-fastidious to grow and an excellent candidate for investigations requiring large amounts of cell material.

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CHAPTER 3: COMPARATIVE STUDY OF PHAGOCYTOSIS IN *SPIRONUCLEUS VORTENS* AND *GIARDIA INTESTINALIS*

3. 1 ABSTRACT

Understanding of transmembrane nutrient transport systems of unicellular parasites is critical in defining host-parasite relationships. Endocytotic processes have thus been extensively studied in parasitic species such as *Giardia intestinalis* and *Trichomonas vaginalis*, but to date, no information is available on endocytotic mechanisms of *Spiroucleus vortens*, an economically important parasite of tropical fish. In this study, flow cytometric and confocal scanning laser microscopic investigations of phagocytosis were conducted in *S. vortens*, using Streptavidin-coated quantum dots and fluorescently labelled bacteria as substrates. Quantum dot uptake by *S. vortens* indicated highly efficient, rapid phagocytosis of these particles, however, phagocytosis of live bacteria by trophozoites did not occur in Keister's modified, TY-I-S33 culture medium.

3.2 INTRODUCTION

Spirotrunculus vortens is a small, eukaryotic flagellate, which is found in the intestine of freshwater tropical fish and is the suspected causative agent of hole-in-the-head disease in cichlids (Poynton, Fraser et al. 1995; Paull and Matthews 2001). Information regarding this host-parasite system is scarce and mechanisms for parasite nutrition within the host are not yet characterized. Specifically, it is unknown whether this parasite is capable of phagocytosis, the process by which cells ingest solid particles, such as bacteria. Phagocytosis is a crucially important aspect of nutrition in most free-living protists, but the ability to ingest solid particles is not always present in parasitic protists, as they often rely on the host to provide digested, dissolved nutrients. For instance, *Trichomonas vaginalis*, an epithelial parasite, is capable of phagocytosis (Garcia-Tamayo, Nunez-Montiel et al. 1978; Street, Wells et al. 1984; Petrin, Delgaty et al. 1998), and obtains many macromolecules through ingestion of host cells and bacteria (Heine and McGregor 1993), whereas *Giardia intestinalis*, a parasite of the intestinal tract, can only perform pinocytosis (Gillin, Reiner et al. 1991; McCaffery and Gillin 1994; Lanfredi-Rangel, Attias et al. 2003; Marti, Regos et al. 2003; Hehl and Marti 2004; Hernandez, Castillo et al. 2007), which enables uptake of much smaller volumes of pre-digested, dissolved nutrients. The presence of phagocytosis may therefore be considered an indication of the degree of nutritional dependency of the parasite to the host. Phagocytosis is also a known virulence factor in *T. vaginalis* and *Entamoeba histolytica* (Benchimol and de Souza 1995; Voigt and Guillen 1999; Voigt, Olivo et al. 1999; Nozaki and Nakada-Tsukui 2006), and is therefore directly relevant to the study of *S. vortens*, as the triggers and mechanisms for virulence in this opportunistic pathogen are poorly understood.

Traditionally, phagocytosis is investigated using bacteria or latex beads, which must be labelled with a fluorophore, so uptake can be monitored via flow cytometry or fluorescence microscopy (Avery, Harwood et al. 1995; Avery, Lloyd et al. 1995). Typical organic fluorescent labels are subject to photobleaching (Bernas, Zarebski et al. 2004; Kolin, Costantino et al. 2006; Muthu, Gryczynski et al. 2007; Satsoura, Leber et al. 2007; Muthu, Gryczynski et al. 2008), and fluorescence spectra often overlap with that of the intrinsic cell fluorescence (Aubin 1979; Benson, Meyer et al. 1979; Eng, Lynch et al. 1989; Billinton and

Knight 2001; Rocheleau, Head et al. 2004; Yang, Li et al. 2008). Such limitations greatly restrict the applications of fluorescent-based cell imaging techniques. However the recent development of colloidal nanoparticles, or quantum dots, may solve these problems. Indeed, these particles possess remarkable fluorescence properties, and in recent years, they have been employed with great success in most areas of biological imaging (Smith and Nie 2004; Gao, Yang et al. 2005; Michalet, Pinaud et al. 2005; Zhou and Ghosh 2007; Smith, Duan et al. 2008). They are especially useful for studying intracellular processes at the single-molecule level, high resolution cellular imaging (Jaiswal, Goldman et al. 2004) and long-term observation of cellular trafficking (Kloepfer, Mielke et al. 2003; Pellegrino, Parak et al. 2003; Jaiswal, Goldman et al. 2004). The basis for that success is that quantum dots generate a much stronger signal than conventional fluorophores (Dubertret, Skourides et al. 2002; Ballou, Lagerholm et al. 2004) and have very broad excitation spectra, combined with narrow emission bands (Alivisatos, Johnsson et al. 1996). These highly specific emission properties circumvent the problem of overlap with the target autofluorescence signal. The fluorescence of quantum dots is also completely resistant to photobleaching (Lacoste, Michalet et al. 2000; Jaiswal, Goldman et al. 2004), a major problem in the fluorescent labelling of biological samples. This permits the use of higher laser power and allows for much longer exposure in extended experiments, which in turn facilitates the generation of images with higher quality resolution (Tokumasu and Dvorak 2003).

In this study, streptavidin-coated quantum dots were employed to investigate the presence of phagocytotic uptake in *S. vortens*, using *G. intestinalis* as a negative control. In order to assess the efficiency of quantum dots in the monitoring of phagocytosis, bacteria hybridised with a DNA probe labelled with a conventional fluorophore (FISH) were also used.

3.3 MATERIAL AND METHODS

Organisms and cultures. *Spiromucleus vortens*, ATCC 50386, was obtained from Prof. J. Kulda (Charles University, Prague, Czech Republic) and cultured axenically at 20°C in Keister's modified TYI-S-33 medium as described in Chapter 2. Log phase cultures were

harvested by centrifugation at 800 g for 3 min at room temperature in a bench centrifuge (MSE minor), washed twice in phosphate buffer saline (pH 6.9, PBS) and resuspended in the same buffer containing 10 mM glucose. *Giardia intestinalis*, JKH strain, was a gift from Dr Timothy Paget (Medway, School of Pharmacy, UK) and was isolated by Victoria Hough. Trophozoites were cultured axenically at 37°C in Diamond's modified medium containing per L: tryptone (BBL), 20 g; yeast extract (oxoid), 10 g; glucose, 5 g; arginine, 1.06 g, NaCl, 1 g; K₂HPO₄, 1 g; KH₂PO₄, 0.6 g; cystein, 1 g; ascorbic acid, 0.2 g; ammonium ferric citrate, 22.8 mg; bovine bile, 1 g; distilled water, 840 ml; heat inactivated newborn calf serum, 100 ml. pH was adjusted to 6.9-7 prior to filter sterilization (0.22 µm pore size). After 10 min incubation of the culture tubes on ice, detached trophozoites were harvested by centrifugation (800 g, 5 min), at 4°C in a Beckman Coulter Avanti J-E centrifuge (Fullerton, California, USA).

Flow cytometry. Approximately 5×10^4 log-phase trophozoites of *S. vortens* or *G. intestinalis* were harvested by centrifugation as described above, washed twice in phosphate buffer saline (pH 6.8, PBS) and resuspended in 200µl PBS (pH 6.8). Cells were then incubated with streptavidin coated Quantum dots (dia. 15 nm, final concentration 1µg. ml⁻¹) for varying time intervals, at 25°C for *S. vortens* and 37°C for *G. intestinalis*. Quantum dots were a gift from Prof Huw Summers and Dr Dan Matthews (Cardiff School of Physics). The cells were then fixed in 3.5% (v/v) formaldehyde and washed thoroughly in PBS. In order to avoid clumping, cells were filtered through 40 µm gauze strainers (Becton Dickinson) and the distribution of fluorescence signal within the cell population was analysed by flow cytometry, using a FACS Canto flow cytometer (Becton Dickinson), equipped with the FACS DIVA v 5.0.3 software (Becton Dickinson). A gated subpopulation of 4×10^4 cells of homogenous size and granularity (Fig. 3. 1.) was selected and the intensity threshold for a positive signal was established at 10^3 area per height (APH) by comparing unlabelled cells (Fig. 3. 2A) with cells incubated with quantum dots (Fig. 3. 2B). The population heterogeneity of fluorescence emission intensity in this subpopulation at 580 nm was then plotted

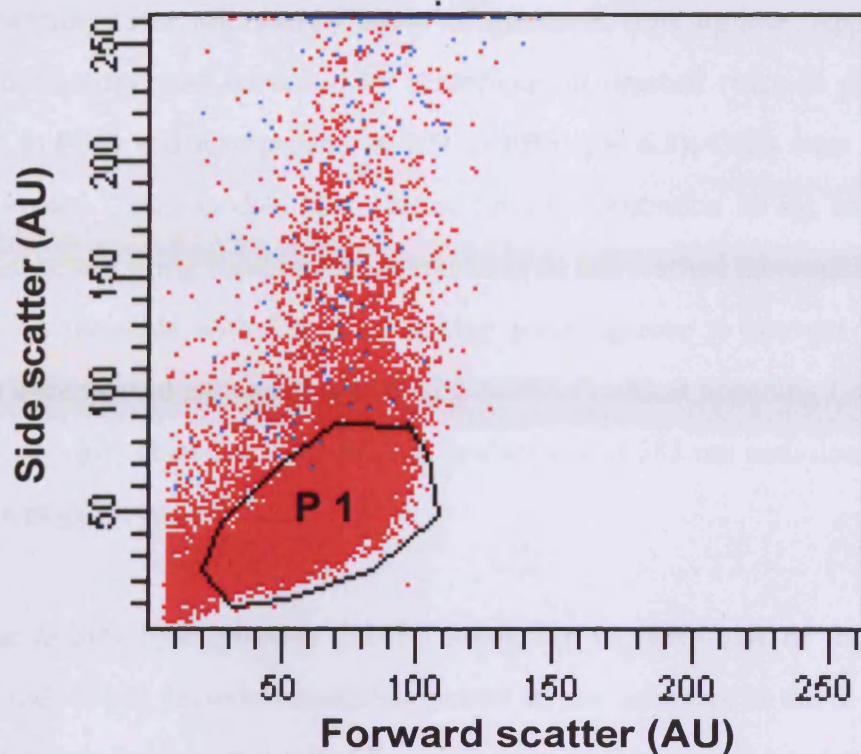


Fig. 3. 1. Selection of a homogenous cell subpopulation (P1) within sample using intensity of forward scatter and area of side scatter as indications of size and granularity, respectively. AU= arbitrary unit.

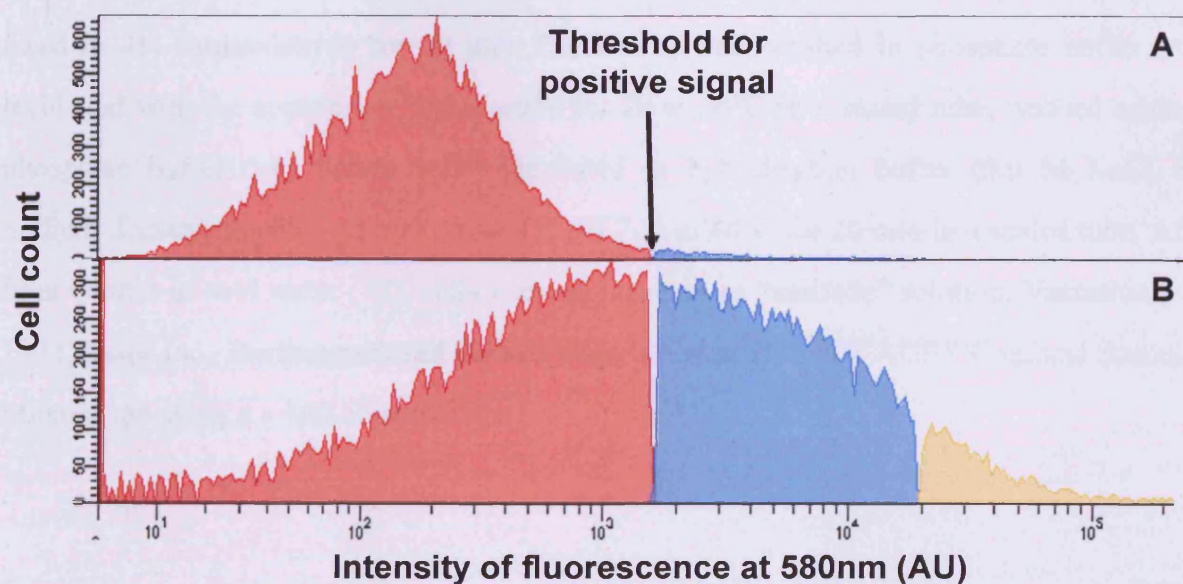


Fig. 3. 2 Determination of threshold for positive fluorescence emission from (A) fluorescence of unlabelled cells and (B) cells incubated with quantum dots for 8 min. AU= arbitrary unit.

Confocal scanning laser microscopy assay of quantum dots uptake. Approximately 10^5 log-phase trophozoites were harvested by centrifugation, washed twice in phosphate buffer saline (pH 6.8, PBS) and resuspended in 200 μ l PBS (pH 6.8). Cells were incubated with streptavidin-coated Quantum dots (dia. 15 nm, final concentration 50 ng. ml⁻¹) for specific time intervals, before being fixed in 3.5% formaldehyde and washed thoroughly in PBS. Cells were mixed on the slide with 2%, low melting point agarose to prevent movement and preparations were viewed with a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope using a $\times 63$ or $\times 100$ objective, with 405 nm excitation and 585 nm emission. *G. intestinalis* was used as a negative control.

Fluorescence *in situ* hybridisation (FISH) assay for phagocytosis of live bacteria. The unidentified rod-shaped bacteria sometimes present in low numbers in the *S. vortens* ATCC culture (see Chapter 2) were maintained in pure culture on Tryptone Soya Agar (TSA, Oxoid Ltd) at 20°C and log-phase, axenic cultures of *S. vortens* were incubated with live rod-shaped bacteria in culture medium for 24h. Whole-cell hybridisation with a fluorescein-labelled Eubmix probe, which targets the conserved region of the 16S rDNA of eubacteria, was then employed to detect the presence of intracellular bacteria. Following the method of Amann *et al.* (1990), cells were harvested as described above, washed ($\times 3$) in phosphate buffer and fixed in 4% formaldehyde for 30 min. Cells were then washed in phosphate buffer ($\times 3$), incubated with the appropriate DNA probe for 2h at 46°C in a sealed tube, washed again in phosphate buffer ($\times 3$) before being incubated in hybridisation buffer (0.9 M NaCl, 0.1 Sodium dodecyl sulphate, 20mM Tris-HCl, pH 7.2) at 48°C for 20 min in a sealed tube. After final washes in cold water ($\times 3$), cells were mounted in an “antifade” solution, Vectashield H-100 (Vector Inc., Burlingame) and viewed under a Leica TCS SP2 AOBS Confocal Scanning Microscope using a $\times 100$ objective.

3. 4 RESULTS

3. 4. 1 Flow cytometry

Figure 3. 3 shows that in *Spironucleus vortens* uptake of quantum dots occurred in a small proportion of the cell population (4.7%) after an incubation of 2 s. That proportion of positive cells remained nearly constant for 30 s, increased sharply to 19.2% after 2 min, before stabilising after 8 min and reaching 33.2% after 1h. In *Giardia intestinalis*, the proportion of positive cells was much lower, 1.7% at the highest, and remained invariant for the different lengths of incubation.

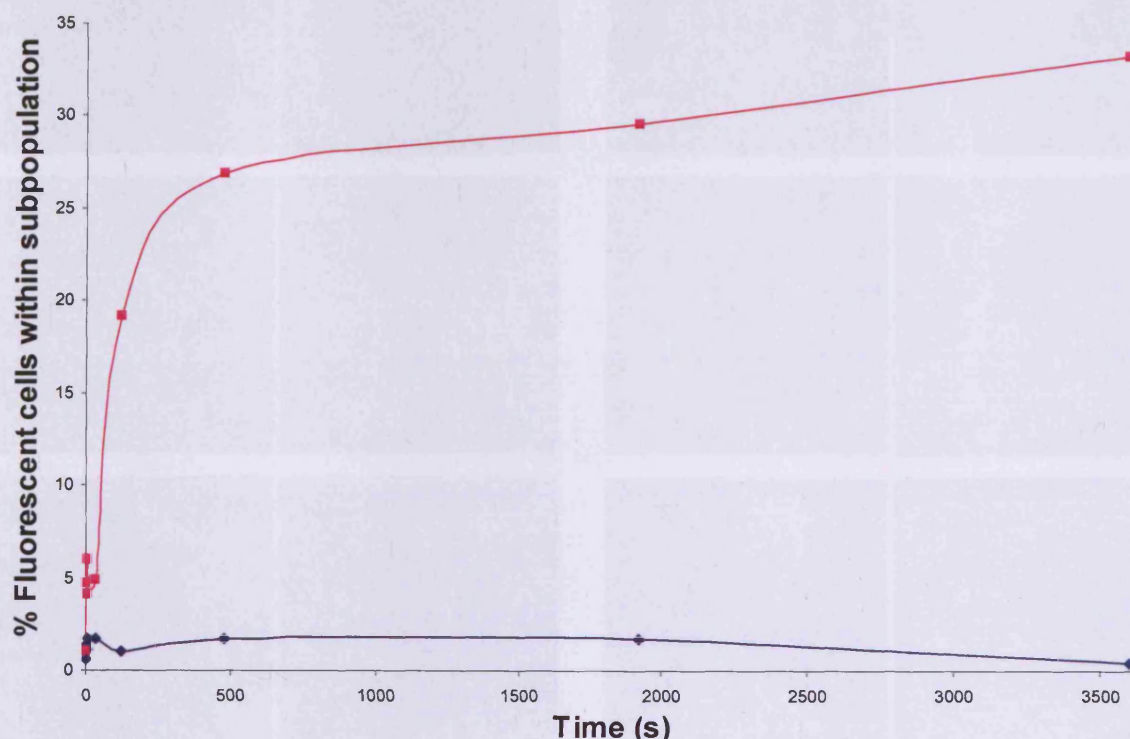


Fig. 3. 3 Comparative study of quantum dots uptake in *Spironucleus vortens* and *Giardia intestinalis*. Log-phase trophozoites were incubated with streptavidin coated quantum dots for 0 s, 2 s, 4 s, 8 s, 32 s, 2 min, 8 min, 32 min and 1h and percentage of fluorescent cells in gated homogenous populations of 4×10^4 cells were measured by flow cytometry (FACS Canto flow cytometer, Becton Dickinson) and plotted against length of incubation.

3. 4. 2 Confocal scanning laser microscopy assay of quantum dots uptake

S. vortens demonstrated rapid, time-dependent uptake of quantum dots (Fig. 3. 4.). Initial uptake occurred within 2 s of incubation and was restricted to the vicinity of the flagellar pockets (Fig. 3. 4A). Dots were then rapidly distributed in a large number of small vacuoles located at the periphery of cells (Figs. 3. 4B & C), and concentrated in a smaller number of larger vacuoles (Fig. 3.4D), before disappearing from the cells (3. 4E & F). The presence of intracellular quantum dots was never observed in *G. intestinalis* (Fig. 3. 5.)

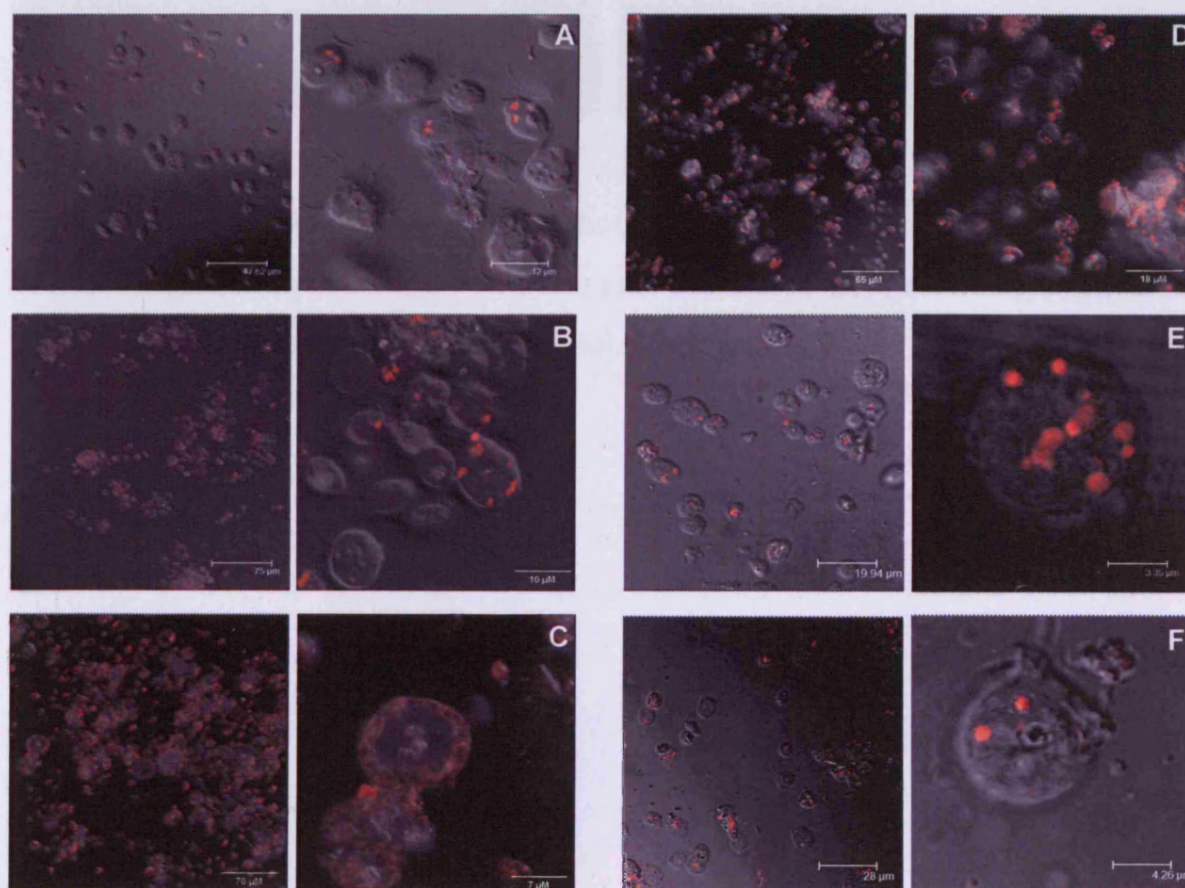


Fig. 3. 4. Confocal scanning laser micrographs overlayed on differential interference contrast images of *Spironucleus vortens* after A) 2s B) 30s C) 1 min D) 3 min E) 10 min and F) 20 min incubations with streptavidin-coated quantum dots, followed by fixation in 3.5% formaldehyde. Paired micrographs represent a low (left panel), and higher (right panel) magnification of the same preparation at a given time point.

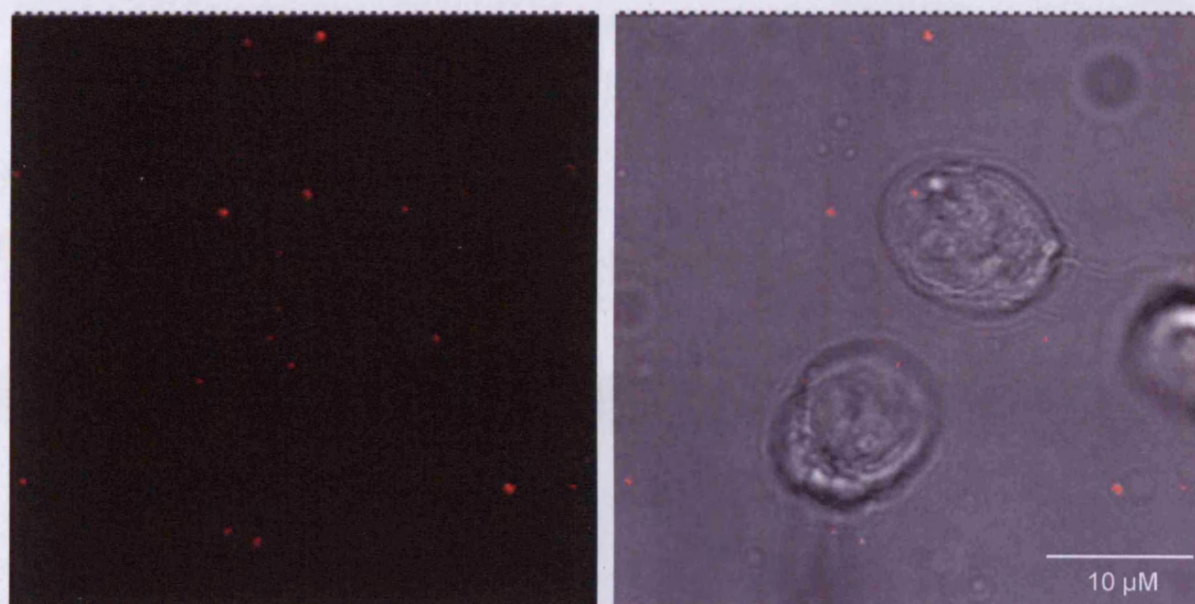


Fig. 3. 5. Confocal scanning laser micrographs (left) and overlay with differential interference contrast images (right) of *Giardia intestinalis* after 10min incubation with streptavidin-coated quantum dots, followed by fixation in 3.5% formaldehyde.

3. 4. 3 Fluorescence in situ hybridisation (FISH) assay for phagocytosis of live bacteria

The 16S rDNA Eubmix probe hybridised with bacterial DNA, revealing the presence of a rod-shaped bacteria that had been added to cultures in the cell suspension of *S. vortens*. However, no intracellular labelling was observed in *S. vortens* trophozoites (Fig. 3. 6) even after exposure for 24h in a mixed culture.

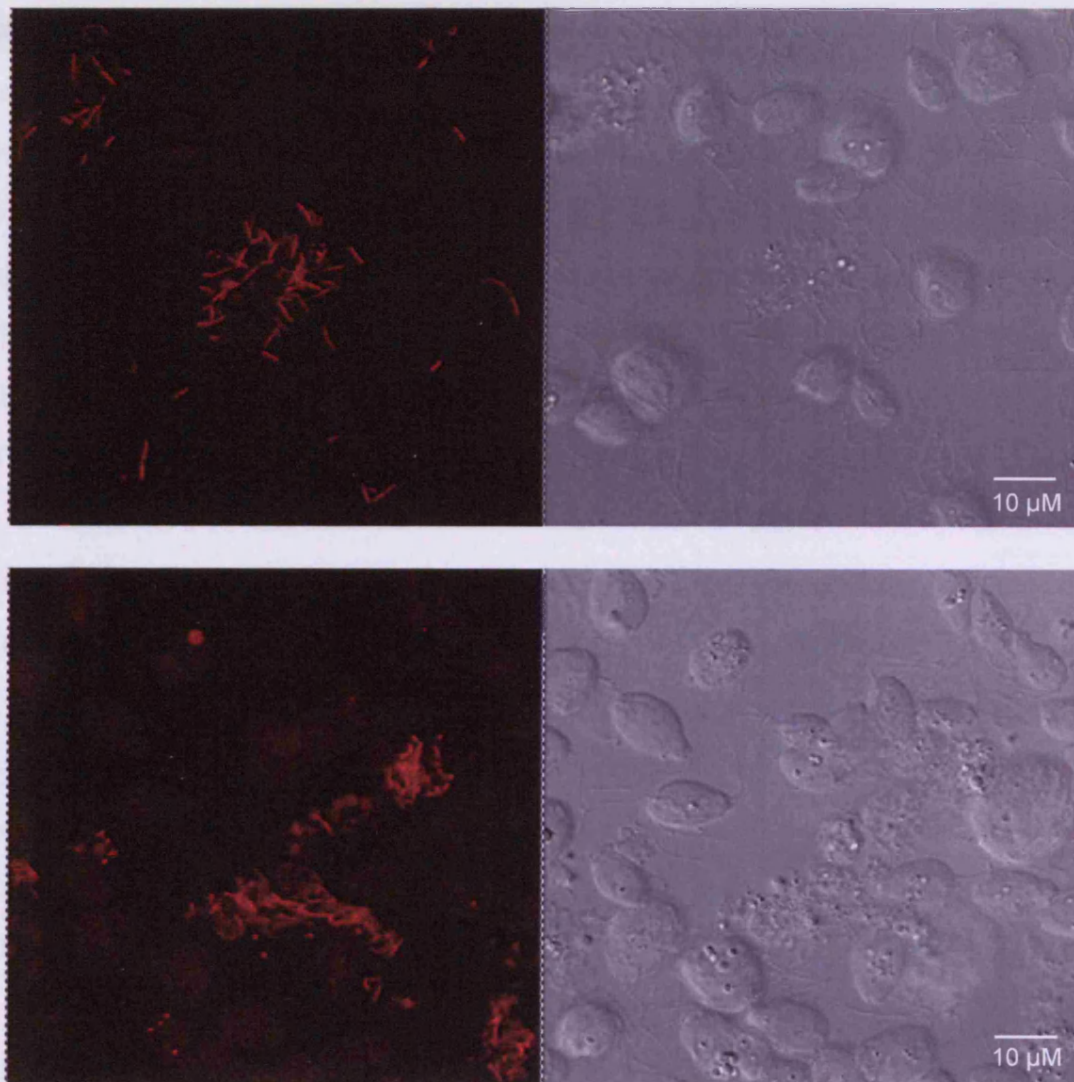


Fig 3. 6. Confocal scanning laser micrographs (left) and differential interference contrast images (right) obtained using a 514 nm laser at 55.70%, combined with 620 V (A) and 652 V (B) detection sensitivity and a showing fluorescence in situ hybridisation of a eubacterial 16S rDNA probe (Eubmix) in *Spironucleus vortens* cultures incubated with an unidentified bacterial contaminant for 24 h.

3. 5 DISCUSSION

Spironucleus vortens is capable of rapid uptake of quantum dots. Flow cytometric analysis of fluorescence in washed cell suspensions incubated with quantum dots revealed a low-level uptake (4.9%) of dots within 2 s of incubation and a significant proportion of the cell population (19.2%) was positive for the presence of intracellular dots after 2 min incubation (Fig. 3. 3.). In contrast, no significant uptake of the dots was observed in *Giardia intestinalis* (Fig. 3. 3.). This organism is known to be incapable of phagocytosis and to rely exclusively on pinocytosis, and other plasma membrane mediated active and passive transport systems for nutrition (Gillin, Reiner et al. 1991; McCaffery and Gillin 1994; Lanfredi-Rangel, Attias et al. 2003; Marti, Regos et al. 2003; Hehl and Marti 2004; Hernandez, Castillo et al. 2007). The fact that quantum dots were undetectable in *G. intestinalis* suggests that even these small (15 nm diameter) particles cannot be transported by such transport systems, thereby confirming that uptake in *S. vortens* is most likely mediated by phagocytosis.

Confocal scanning laser microscopic examination of quantum dot uptake in washed cell suspensions confirmed that *S. vortens* is capable of rapid phagocytosis of quantum dots, and revealed that the initial uptake is restricted to the vicinity of the flagellar pockets (Fig. 3. 4A). This is consistent with what is known of phagocytosis in most protozoan flagellates, in which phagocytosis occurs at one site on the plasma membrane, via a specialised organ, the cytostome (Brugerolle, Joyon et al. 1973; Brugerolle 1975). Following initial uptake at the flagellar pockets (Fig 3. 4A), quantum dots were distributed in many small vacuoles (Figs. 3. 4B & C), which appeared to fuse and form larger vacuoles after 3 min (Fig 3. 4D). After 10 min, the amount of intracellular fluorescence decreased (Fig 3. 4E). Because quantum dots are highly resistant to photobleaching, the decrease in intracellular fluorescence could only be caused by their excretion from the cells. The excreted dots appeared to no longer be taken up by the cells, as intracellular fluorescence continued to decrease (Fig. 3. 4F). The most likely explanation is that cells had digested the protein coating of the phagocytosed quantum dots, so that after excretion, they were no longer recognised as edible particles and therefore failed to trigger phagocytosis. Indeed, in this experiment, cells were incubated with a rather small amount of quantum dots. Intracellular concentrations of the fluorescent particles would

therefore have decreased rapidly, as digested dots were being excreted, but failed to be replaced, due to the reduced availability of the protein-coated dots in the surrounding buffer. In the flow cytometric analysis of quantum dots uptake, a higher concentration of quantum dots was employed, and no decrease in intracellular quantum dots concentration was observed after 1h of incubation (Fig 3. 1C), which tends to confirm that particles were being excreted after digestion of their protein coating. In order to confirm this theory, the confocal experiment should be repeated using a higher concentration of the nanoparticles, and the flow cytometry experiment with a lower concentration. Indeed, the flow cytometry technique gives quantitative information on the proportion of fluorescent cells in a population, rather than on the intensity of fluorescence within individual cells, and it is therefore difficult to directly compare results of both analyses.

Although *S. vortens* proved capable of highly efficient phagocytosis of small (15 nm) particles, no uptake of bacteria (average $0.2 \times 3 \mu\text{m}$) could be detected by fluorescence *in situ* hybridisation, using a bacterial 16S rDNA probe, in cultures inoculated with a live, rod-shaped bacterium for 24h. It is possible that the protist was not able to ingest the relatively large bacteria or that the rod-shaped bacteria possessed defence mechanisms against phagocytosis. In order to investigate this further, future work should include incubation with smaller, heat-killed bacteria. The use of pre-labelled, heat-killed bacteria would also circumvent potential problems with the permeability of the protozoan cuticle to the DNA probe. Indeed, the FISH protocol used in this study was developed by Amann, Krumholz et al. (1990) for the detection of endosymbiotic bacteria in free-living protozoa, but has not been optimised for *S. vortens* and its cuticle may therefore be more impermeable to such chemical labels.

Another explanation for the apparent lack of bacterial phagocytosis in *S. vortens* is that this process may not occur when soluble, digested nutrients are available in the surrounding fluid. Indeed, in this experiment, incubation with bacteria was performed in growth medium, which contains all necessary nutrients. The presence of a nutrient-dependent switch between the uptake of soluble molecules and phagocytosis of solid particles would be extremely interesting, as it would implicate that cells may adapt to environments poor in dissolved nutrients by ingesting host cells or bacteria (Heine and McGregor 1993). Such

adaptations may be crucial to survival outside the gut, in the course of systemic, pathogenic infections. For instance, in the epithelial infections characteristic of hole-in-the-head disease (Paull and Matthews 2001), bacteria and host cells could represent the largest source of nutrients available to the organism. Moreover, phagocytosis of bacteria may even enable survival outside the host, which would be very important during transmission of the parasite. Future work should therefore include incubation of starved trophozoites in buffer with heat-killed or live bacteria, as well as incubation of quantum dots in culture medium.

This study has clearly demonstrated that *S. vortens* possesses a highly efficient phagocytotic apparatus and that when placed in a nutrient-poor environment, trophozoites are capable of rapid scavenging of small particles. Protein coated quantum dots proved to be an attractive alternative to the use of latex beads, as they were readily taken up by cells, and their resistance to photobleaching allowed a reliable assessment of intracellular fluorescence, as well as the generation of high resolution micrographs. It is likely that quantum dots will contribute in further defining the phagocytotic process in *S. vortens* and other protists. Indeed, according to their size, these particles are readily synthesised in a range of different colours (Alivisatos et al, 1996; Michalet et al, 2005; Smith et al, 2008; Smith and Nie, 2004), and simultaneous use of several sizes of quantum dots in a time-lapse study, would therefore permit a more in depth description of phagocytosis, both on a spatial and kinetic level. If conjugated to specific antibodies, they could also be used in the fluorescence immunolocalisation of the molecules involved in triggering phagocytotic uptake.

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CHAPTER 4: THE DIPLOMONAD FISH PARASITE *SPIRONUCLEUS VORTENS* PRODUCES HYDROGEN

4. 1 ABSTRACT

The diplomonad fish parasite *Spiroucleus vortens* causes major problems in aquaculture of ornamental fish, whilst the related species *S. barkhanus* affects wild and farmed salmonids, resulting in severe economic losses in the fish farming industry. The strain of *S. vortens* studied here was isolated from an angelfish and grown in bile supplemented TYIS33 medium. A membrane-inlet mass spectrometer was employed to monitor, in a closed system, O₂, CO₂ and H₂. When introduced into air saturated buffer, *S. vortens* rapidly consumed O₂ at the average rate of 62 ± 3.89 nmoles min⁻¹ per 10⁷ cells and CO₂ was produced at 75.30 ± 11.20 nmoles min⁻¹ per 10⁷ cells. H₂ production began under microaerophilic conditions ([O₂] = 32.45 ± 15.02 μM) at a rate of 76.80 ± 6.53 nmoles min⁻¹ per 10⁷ cells. H₂ production was inhibited by 61.5% at 150 μM KCN and by 50% at 0.24 μM CO, suggesting that an Fe-only hydrogenase is responsible for H₂ production. Metronidazole (1mM), the compound commonly used to treat fish infected with *Spiroucleus* spp., inhibited H₂ production by 50%, while CO₂ production was not affected. This suggests that, as in the hydrogenosome of *T. vaginalis*, metronidazole is reduced by an enzyme of the H₂ pathway, thus competing for electrons with H⁺.

4. 2 INTRODUCTION

Hydrogen production is rare within the eukaryotic kingdom and, to date, has only been observed in a few anaerobic or microaerophilic organisms including some chytrid fungi, free-living ciliates from anaerobic sediments, rumen ciliates, parabasalids, such as *Trichomonas vaginalis*, as well as some green algae, when growing anaerobically in light (Paul, Williams et al. 1990; Brugerolle and Muller 2000; Cammack, Horner et al. 2003). In these anaerobic (or microaerophilic) organisms, protons serve as terminal electron acceptors, forming molecular H₂. This process contributes to the reoxidation of NAD[P]H in the absence of a conventional respiratory chain. The principal site for energy generation in anaerobic (and microaerophilic) eukaryotes is the cytosol, however, in most H₂ generating protists, the conversion of pyruvate to acetate takes place in a special redox organelle, the hydrogenosome (Lindmark and Muller 1973; Paul, Williams et al. 1990; Hackstein, Akhmanova et al. 1999; Mukherjee, Brown et al. 2006; Lantsman, Tan et al. 2008; van Grinsven, Rosnowsky et al. 2008). Hydrogenosomes are 0.4 to 0.6 µm dia. in size, bounded by two membranes. They are believed to have been derived from mitochondria during the evolutionary adaptation to microaerophilic or anaerobic environments. They occur in several non-related eukaryotic lineages, which are often closely related to corresponding aerobic organisms, from which they are derived, suggesting that they have evolved repeatedly during evolution (Paul, Williams et al. 1990; Embley, Finlay et al. 1995; Horner, Hirt et al. 1996; Biagini, Finlay et al. 1997; Embley and Hirt 1998; Horner, Hirt et al. 1999; Horner, Foster et al. 2000; Embley, van der Giezen et al. 2003a; Embley, van der Giezen et al. 2003b; Embley 2006).

The enzymes involved in H₂ generation in eukaryotes are pyruvate:ferredoxin oxidoreductase (PFOR), ferredoxin (or flavodoxin) and Fe-only hydrogenases (Paul, Williams et al. 1990; Horner, Hirt et al. 1999; Horner, Foster et al. 2000; Cammack, White et al. 2003; Hrdy, Hirt et al. 2004). The latter contain a commonly recognised iron-sulphur motif. Short sequences with homology to Fe-only hydrogenases are also found in most aerobic eukaryotes, including humans *narf* gene family, although none of these aerobic organisms actually produce H₂ (Horner, Hirt et al. 1999; Horner, Foster et al. 2000; Happe and Kaminski 2002). This suggests an ancient and crucial role for this protein family in eukaryotes. Some authors

hypothesize that H₂ syntrophy was one of the main drivers for the endosymbiosis that gave rise to mitochondria and that H₂ generating enzymes were present in the original eukaryote, thereby providing its endosymbiont with the means of CH₄ generation (Martin and Muller 1998; Horner, Hirt et al. 1999; Horner, Foster et al. 2000; Cammack, Horner et al. 2003). Enzymes of the H₂ pathways would have subsequently been recruited during the evolutionary modification of aerobes to anaerobic lifestyles and the evolution of the hydrogenosome. Alternatively, some or all of these enzymes may have been transferred by lateral gene transfer from bacterial sources. Fe-hydrogenases do not appear to be monophyletic in all H₂ generating organisms, whereas PFOR sequences are, which is surprising in organisms originating from such diverse lineages: this reinforces the hypothesis of an ancient origin for PFORs (Horner, Hirt et al. 1999; Horner, Foster et al. 2000).

The microaerophilic diplomonad parasite *Giardia intestinalis*, constitutes a significant exception among H₂ generating organisms, as it lacks hydrogenosomes and the site of H₂ production is cytosolic (Lloyd, Ralphs et al. 2002). Based on their rRNA phylogenies and also due to their apparent lack of organelles, diplomonads were assigned to basal eukaryotic taxa (Cavalier-Smith and Chao 1996). However, evidence that genes of mitochondrial origin are present in diplomonads suggests that these parasites are not actually primitive and may have lost their mitochondria in a secondary event (Embley and Hirt 1998; Morin 2000; Lloyd and Harris 2002).

The Hexamitidae family includes only a few free-living forms but with a majority of parasitic species infecting a wide range of animals, from insects, reptiles, fish and birds to mammals (Slavin and Wilson 1953; Kunstyr 1977; Sebesteny 1979; Januschka, Erlandsen et al. 1988; Harper 1991; Kunstyr, Poppinga et al. 1993; O'Brien G, Ostland et al. 1993; Whitehouse, France et al. 1993; Poynton, Fraser et al. 1995; Baker, Malineni et al. 1998; Pennycott 1998; Sterud 1998; Paull and Matthews 2001; Sterud and Poynton 2002; Cooper, Charlton et al. 2004; Poynton, Fard et al. 2004; Wood and Smith 2005; Saghari Fard, Weisheit et al. 2007). Some of these species can also be opportunistic pathogens. Thus, the fish parasite *S. vortens* (Poynton, Fraser et al. 1995) causes severe systemic infections in several species of ornamental fish and has been associated with hole-in-the-head disease in cichlids, a disease of considerable economic impact in the aquarium trade (Paull and

Matthews 2001). Although an Fe-hydrogenase sequence has been identified in the genome of *S. barkhanus* (Horner, Foster et al. 2000; Nixon, Field et al. 2003), a related fish pathogen which causes devastating systemic infections in salmonids, H₂ production within the hexamitid family has not yet reported. In this study, membrane inlet mass spectrometry was employed to investigate the presence of an H₂ production pathway in *S. vortens*.

4. 3 MATERIALS AND METHODS

Origin of samples. *Spironucleus vortens*, ATCC 50386, were grown in TY-I-S33 medium as described in Chapter 2, log phase cultures were harvested by centrifugation at 800 g for 2 min at room temperature in a bench centrifuge (MSE minor), washed twice in phosphate buffer saline (pH 6.8, PBS), and resuspended in the same buffer containing 10 mM glucose.

Mass spectrometry. Full methodology is described in Lloyd & Scott (1982) and Lloyd *et al.* (1992). Briefly, log phase trophozoites were harvested by centrifugation (800g, 3 min), washed (x2) in PBS (pH= 6.8), resuspended in 5 ml of air saturated PBS and introduced into a 5 ml perspex, closed, reaction vessel using a Hamilton syringe. Dissolved gases were measured in the cell suspension, using a Hal series quadrupole mass spectrometer (Hiden Analytical) fitted with a 10 cm, 1.6 mm dia. stainless steel probe covered by a gas-permeable polymer membrane (1.56 mm outside dia., 0.5 mm internal dia., 5 cm length). The inlet orifice was 100 µm dia., and the membrane used was silicone rubber catheter tube. Cells were stirred continuously at 150 r.p.m. and maintained at 20°C by circulating thermostatically maintained water around the reaction vessel. Viability of cells was assessed at the end of experiments by investigating cell motility, using an Olympus light microscope equipped with a X10 objective. Mass to charge ratios (m/z) used to measure the concentrations of H₂, ethanol, O₂ and CO₂ were 2, 31, 32 and 44, respectively. Solubilities of H₂ and O₂ at 20 °C were 810 and 1390 µM, respectively (Wilhelm, Battino *et al.*, 1977). CO₂ calibrations employed additions of 3.3 mM NaHCO₃. Ethanol is known to give a signal on the $m/z = 2$ channel, at which H₂ is normally detected. To avoid non-specific detection of ethanol in this channel, both ethanol and H₂ calibrations were performed at $m/z = 2$. Ethanol calibrations

employed stepwise additions of 2 mM C₂H₅OH. Under the conditions employed here, addition of 6 mM ethanol did not cause any increase in the m/z = 2 channel (Fig. 4. 1), thus confirming that this channel is specific for the detection of H₂.

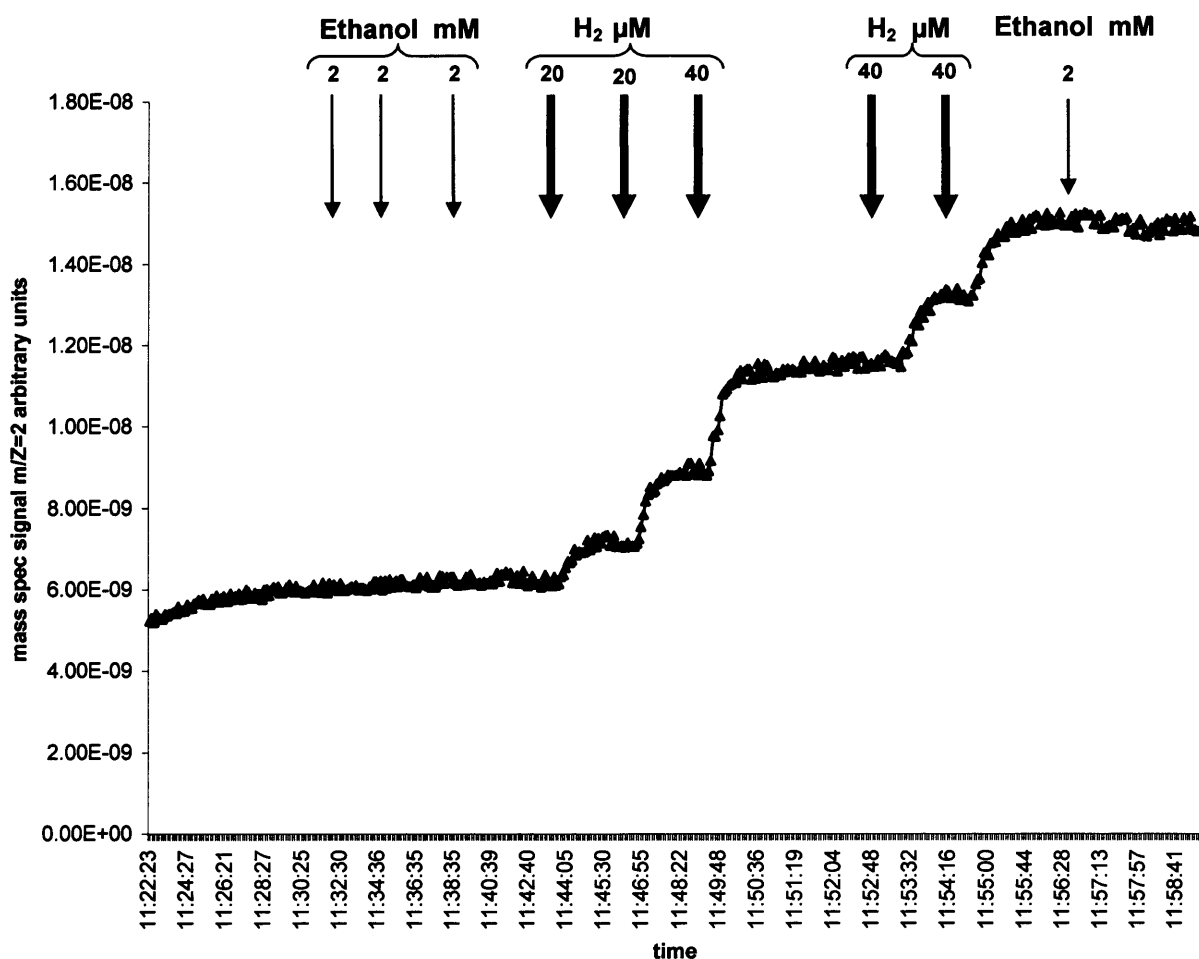


Fig. 4. 1. Calibration curve indicating the specificity of H₂ detection on the m/z = 2 channel of the mass spectrometer. Even 6mM ethanol produced no response.

Inhibitors. Effects of KCN (0.075 mM to 20 mM), CO (0.24 to 5.52 mM), metronidazole (1 mM to 1.5 mM), NaNO₂ (5mM to 10 mM) and iodoacetamide (10 mM) on gas production were investigated. Inhibitors were injected into the reaction vessel at recorded times using a Hamilton syringe.

Statistical analysis. Rates of production for CO₂, O₂ and H₂ were calculated by linear regression analysis using Minitab vs. 13. Measure of variability is given by the standard error.

4. 4 RESULTS

4. 4. 1 Gas metabolism.

When introduced into air saturated buffer, *Spironucleus vortens* rapidly consumed O₂ at an average rate of 62.10 ± 3.89 nmoles min⁻¹ per 10⁷ cells (n = 3). A burst of CO₂ production was observed for the first 3 ± 0.32 min (n = 3) and CO₂ was then produced at the slower rate of 75.30 ± 11.20 nmoles min⁻¹ per 10⁷ cells (n = 3).

H₂ production started only after O₂ consumption had reduced levels of dissolved O₂ to microaerophilic conditions, at an oxygen concentration of 32.45 ± 15.02 μM (n=9), and its rate was 76.80 ± 6.53 nmoles min⁻¹ per 10⁷ cells (n = 4). This slowed after H₂ levels reached high concentrations before altogether stopping as cells lost viability (Fig. 4. 2.), indicated by the loss of cell motility determined by microscopic examination of samples withdrawn from the reaction vessel

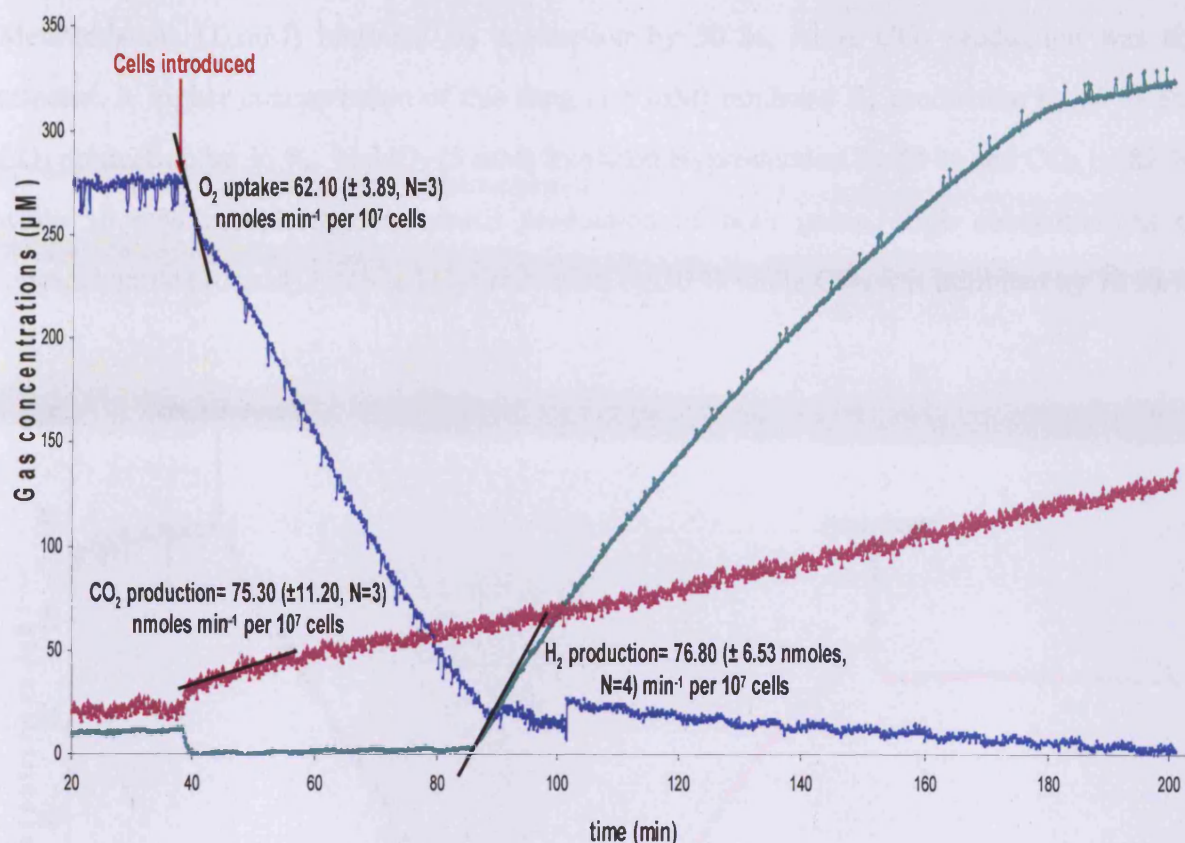


Fig. 4. 2. Representative example of the output of a mass spectrometric experiment measuring gas production in a stirred suspension of *Spironucleus vortens*. Washed organisms (7.4×10^5 cells ml^{-1}) were added to air-saturated in PBS containing 10 mM glucose and introduced into a closed reaction vessel. The temperature was 20°C and the membrane interface of the probe was silicone rubber. The mass spectrometer was programmed to scan mass spectra repeatedly, collecting 128 spectra, before presenting data quasi-continuously at m/z values 2, 32 and 44, corresponding to peak values for H_2 , O_2 and CO_2 , respectively. O_2 consumption, CO_2 production and H_2 production rates were determined by linear regression analysis (minitab 13), at the time points indicated by the drawn tangents. Standard error and number of replicates are shown between brackets. —●— $[\text{H}_2]$ —●— $[\text{O}_2]$ —●— $[\text{CO}_2]/10$

4. 4. 2 Effect of inhibitors.

Cyanide (KCN) inhibited H_2 production by 26 % (at 75 μM) to 61.5 % (150 μM), while CO_2 production was not affected. KCN at 10 mM completely suppressed H_2 production (Fig. 4. 3) CO inhibited H_2 production by 50 % at 0.24 mM without affecting CO_2 production.

Metronidazole (1 mM) inhibited H_2 production by 50 %, while CO_2 production was not affected. A higher concentration of this drug (1.5 mM) inhibited H_2 production by 87 % and CO_2 production by 36 %. $NaNO_2$ (5 mM) inhibited H_2 production by 58 % and CO_2 by 82 %, while 10 mM completely suppressed production of both gases. High concentrations of iodoacetamide (10 mM) inhibited H_2 production by 30 % while CO_2 was inhibited by 72 %.

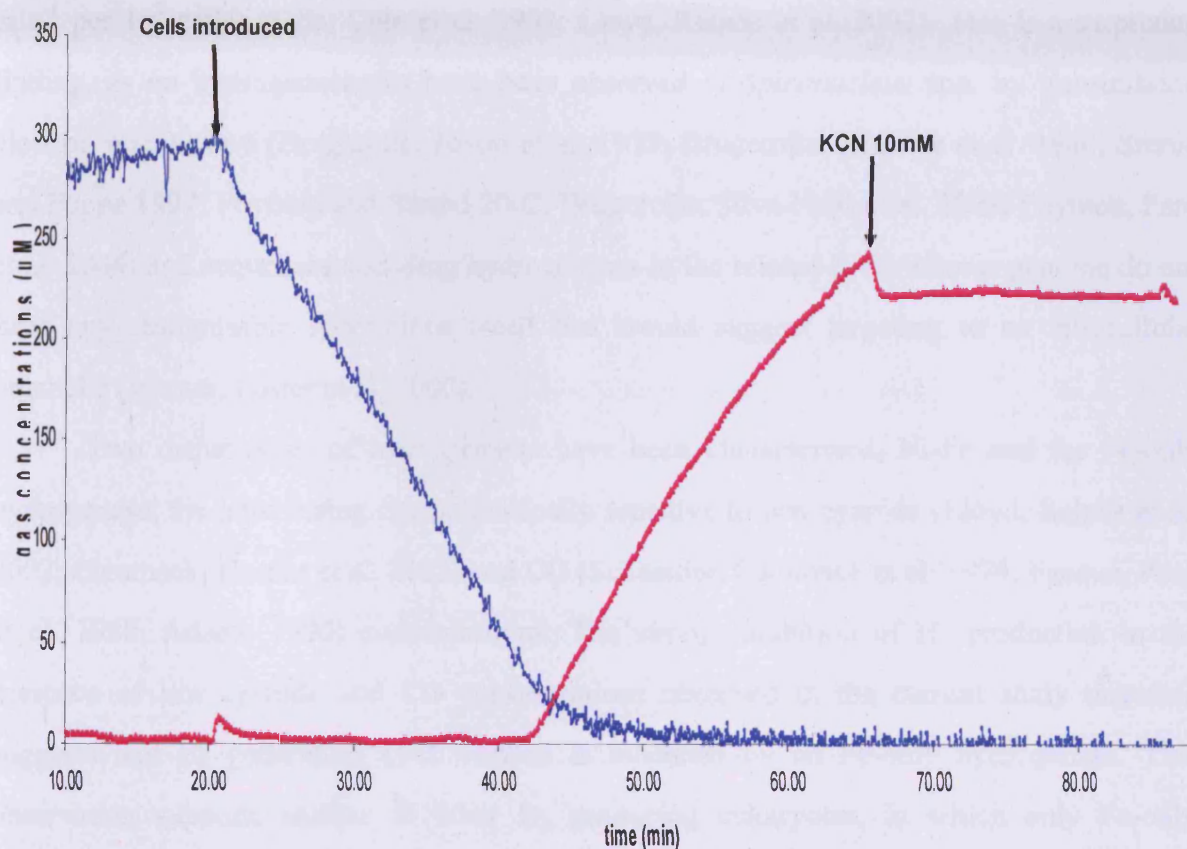


Fig. 4. 3 Effect of KCN on H_2 production in a cell suspension of *Spironucleus vortens*.

Gas production measured in a stirred suspension of *S. vortens*. Approximately 10^6 washed cells ml^{-1} were added to air-saturated PBS containing 10 mM glucose and introduced into a closed reaction vessel. Cyanide (10 mM) was added into the reaction vessel after H_2 production had started. The temperature was $20^\circ C$ and the membrane interface of the probe was silicone rubber. The mass spectrometer was programmed to scan mass spectra repeatedly, collecting 128 spectra, before presenting data quasi-continuously at m/z values 2 and 32, corresponding to peak values for H_2 and O_2 , respectively. — $[O_2]$ — $[H_2]$

4. 5 DISCUSSION

Spironucleus vortens produces H₂ very rapidly (76.80 nmoles min⁻¹ per 10⁷ cells). This rate is much higher than that of *Giardia intestinalis* (2 nmoles/min/10⁷ cells), the only eukaryote reported to produce H₂ without possessing hydrogenosomes and is more similar to that of an organism with well defined hydrogenosomes, *Trichomonas vaginalis* (20 nmoles min⁻¹ per 10⁷ cells) (Ellis, Cole et al. 1992; Lloyd, Ralphs et al. 2002). This is a surprising finding, as no hydrogenosomes have been observed in *Spironucleus* spp. by transmission electron microscopy (Brugerolle, Joyon et al. 1973; Brugerolle, Kunstyr et al. 1980; Sterud and Poppe 1997; Poynton and Sterud 2002; Brugerolle, Silva-Neto et al. 2003; Poynton, Fard et al. 2004) and sequences encoding hydrogenases in the related *S. barkhanus* genome do not have any recognisable N-terminus motif that would suggest targeting to an intracellular organelle (Horner, Foster et al. 2000).

Two major types of hydrogenases have been characterised, Ni-Fe and the Fe-only hydrogenase, the latter being characteristically sensitive to low cyanide (Lloyd, Ralphs et al. 2002; Cammack, Horner et al. 2003) and CO (Schneider, Cammack et al. 1979; Fauque, Peck et al. 1988; Adams 1990) concentrations. The strong inhibition of H₂ production in the presence of low cyanide and CO concentrations observed in the current study therefore suggests that H₂ generation in *S. vortens* is mediated by an Fe-only hydrogenase. This observation supports studies of other H₂ producing eukaryotes, in which only Fe-only hydrogenases have been isolated so far. An Fe-hydrogenase-like sequence has also been identified in the genome of *S. barkhanus*, a related parasitic species that infects salmonids (Horner *et al.*, 2000; Nixon *et al.*, 2003). Genesis of H₂ at a relatively high extracellular oxygen tension (32.45 µM) is, however, uncharacteristic of an Fe-only hydrogenase, the activity of which is extremely sensitive to O₂ (Lloyd, Ralphs et al. 2002; Cammack, Horner et al. 2003). This may indicate the presence of a second type of hydrogenase, or of an extremely effective intracellular O₂ scavenging system.

Metronidazole is the drug of choice in the treatment of anaerobic infections, including giardiasis and trichomoniasis in humans and animals (Zimmer 1987; Ozbilgin, Ozbek et al. 1994; Samour, Bailey et al. 1995; McKeon, Dunsmore et al. 1997; Czeizel and Rockenbauer

1998; Harris, Plummer et al. 2001; Price, Stewart et al. 2006), as well as spironucleosis and hexamitosis in birds and fish (Sangmaneeet and Smith 1999; Philbey, Andrew et al. 2002). This drug is activated when reduced by electron donation from the low redox potential, iron sulphur proteins of anaerobic pathways, and is thus selectively toxic to anaerobic organisms. In *T. vaginalis*, it has been proposed that metronidazole is reduced by the pyruvate:ferredoxin oxidoreductase, and on the ferredoxin of the H₂ production pathway, thus competing for electrons with the hydrogenases, without directly affecting the glycolytic pathways (Ellis, Wingfield et al. 1993; Ellis, Yarlett et al. 1994; Cammack, Horner et al. 2003; Cudmore, Delgaty et al. 2004). In *S. vortens*, 1 mM metronidazole strongly inhibited H₂ production without perceptible effects on CO₂ production, suggesting similarities in the H₂ producing pathways of this organism and the hydrogenosomes of *T. vaginalis*. A hypothetical pathway for H₂ production and probably sites of inhibitor action in *S. vortens* is shown in Figure 4. 4. This simple pathway is based on current knowledge of the mode of action of metronidazole in *Giardia* and *Trichomonas* species, but is probably incomplete. Indeed, recent proteomic studies in *Entamoeba histolytica*, have shown that metronidazole may be activated, not only by ferredoxins, but also by the thioredoxin reductase. Moreover, besides causing DNA damage, the activated drug would also target and bind to several enzymes involved in redox balance, causing a significant drop in non-protein thiol levels (Leitsch, Kolarich et al. 2007),. This could in turn affect the activity of O₂ sensitive enzymes, such as Fe-hydrogenase.

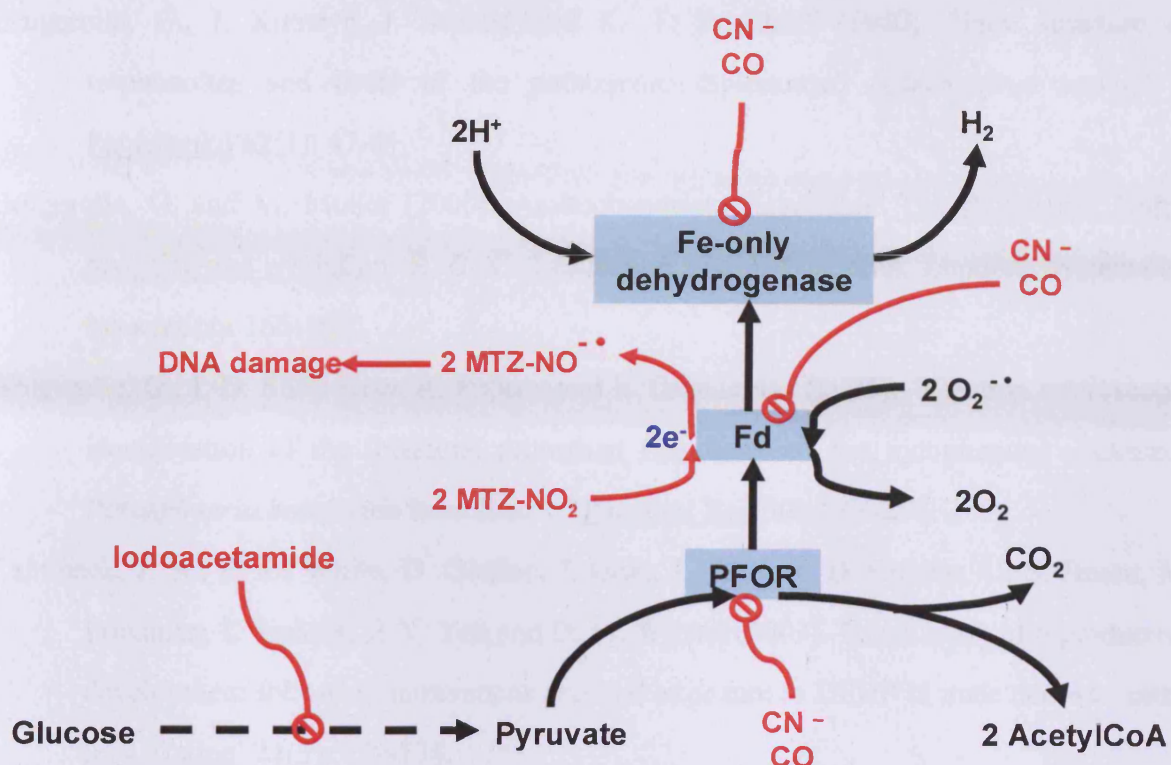


Fig. 4. 4 Hypothetical pathway for energy metabolism and hydrogen production in *Spirotrunculus vortens*. Putative sites for the action of inhibitors are shown in red.

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CHAPTER 5: CARBOHYDRATE AND AMINO ACID METABOLISM OF *SPIRONUCLEUS VORTENS*

5.1 ABSTRACT

The metabolism of *Spironucleus vortens*, a parasitic, diplomonad flagellate related to *Giardia intestinalis*, was investigated using a combination of ^1H NMR, ^{13}C NMR, bioscreen continuous growth monitoring, membrane inlet mass spectrometry and ion exchange chromatography. ^1H NMR and ^{13}C NMR identified the products of glucose-fuelled and endogenous metabolism as ethanol, acetate, alanine and lactate. Mass spectrometric monitoring of gas metabolism in buffered cell suspensions showed that glucose and ethanol could be used by *S. vortens* as energy-generating substrates, but bioscreen automated monitoring of growth in culture medium, as well as NMR analyses, suggested that neither of these compounds are the substrates of choice for this organism. Ion-exchange chromatographic analyses of free amino-acid and amino-acid hydrolysate of growth medium revealed that, despite the availability of large pools of free amino-acids in the medium, *S. vortens* hydrolysed large amounts of proteins during growth. The organism produced alanine and aspartate, and utilized lysine, arginine, leucine, cysteine and urea. However, mass spectrometric and bioscreen investigations showed that addition of these amino acids to the culture medium did not induce any significant increase in metabolic or growth rates. Moreover, NO electrode monitoring revealed that addition of arginine did not generate NO production, rendering the presence of an energy-generating, arginine dehydrolase pathway in *S. vortens* unlikely.

5.2 INTRODUCTION

Spironucleus vortens is a small, amitochondriate, unicellular eukaryote, which is distantly related to *Giardia* and *Trichomonas* species (Jorgensen and Sterud 2007), and is capable of colonising a wide range of different habitats. Indeed, while it is primarily described as a gut commensal of freshwater fish, it is also found in the kidneys, liver, spleen and skin lesions of fish suffering from hole-in-the-head disease (Nigrelli and Hafter 1947; Ferguson and Moccia 1980; Bassleer 1983; O'Brien G, Ostland et al. 1993; Poynton, Fraser et al. 1995; Paull and Matthews 2001). Trophozoites are also known to survive in freshwater for over 10 h (see Chapter 2.1). This seemingly simple, eukaryotic parasite must therefore be able to utilize different nutrient resources, which hints at a highly versatile metabolism.

The closely related free-living *Hexamita inflata* appears capable of utilising a range of carbohydrates and amino acids (Biagini, McIntyre et al. 1998), and the parasitic *Trichomonas vaginalis* and *Giardia intestinalis*, although they colonise distinctively less versatile environments, have been shown to demonstrate a certain amount of metabolic plasticity (Lindmark 1980; Jarroll, Manning et al. 1989; Lloyd and Paget 1991; Schofield and Edwards 1991; Brown, Upcroft et al. 1998; Petrin, Delgaty et al. 1998). Indeed, although they can most efficiently utilise glucose or glucose polymers, these parasites possess very active proteinases (Lockwood, North et al. 1984; Lockwood, North et al. 1987; Lockwood, North et al. 1988; Lockwood and Coombs 1991b; North 1991a&b; Mallinson, Lockwood et al. 1994; Williams and Coombs 1995), which enables them to hydrolyse proteins when carbohydrates are scarce. This process is demonstrated in *Trichomonas* cultures, as products of amino acid catabolism such as indole and dimethylsulphide can be isolated from conditioned growth medium (Lloyd, Lauritsen et al. 1991). Many other protozoan parasites are known to similarly resort to incomplete oxidation of amino acids for energy generation; alanine or proline being the most common end-products (Lockwood and Coombs 1991a; Melhorn 2009). An energy-generating arginine dihydrolase pathway, which results in formation of ornithine and putrescine with concomitant production of nitric oxide, has also been extensively characterised in *T. vaginalis* and *G. intestinalis* (Linstead and Cranshaw 1983; Schofield, Costello et al. 1990; Schofield,

Edwards et al. 1992; Yarlett, Martinez et al. 1996; Yarlett, Martinez et al. 2000; Harris, Goldberg et al. 2006).

While the extent of metabolic versatility is well characterised in *T. vaginalis* and *G. intestinalis*, nothing is currently known of the metabolism of *S. vortens*. In order to investigate the basic metabolic characteristics of this organism, carbohydrates and amino acid metabolism were investigated using membrane inlet mass spectrometry and ion exchange chromatography. Growth upon a range of substrates was then studied by automated optical density monitoring, while products of endogenous and carbohydrate metabolism were identified using proton and ^{13}C NMR.

5.3 MATERIAL AND METHODS

Organisms and Cultures. *Spironucleus vortens*, ATCC 50386, were grown in TY-I-S33 medium as described in Chapter 2. *Trichomonas vaginalis*. C1 (ATCC 30001) cultures were grown at 37°C in TYM medium containing per litre: trypticase (BBL), 20 g; yeast extract (Oxoid), L-cysteine. HCl (Sigma), 1 g; ascorbic acid sodium salt (Fluka), 0.2 g; K_2HPO_4 (Merck), 0.8 g; KH_2PO_4 (Merck), 0.8 g; heat-inactivated horse serum (Difco), 10 ml. The pH was adjusted to 6.2 with NaOH prior to autoclaving. Log phase cultures were harvested by centrifugation at 800 g for 2 min at room temperature in a bench centrifuge (MSE minor).

Membrane inlet mass spectrometry. The effect of various metabolites on gas metabolism was investigated by membrane inlet mass spectrometry as described in Chapter 3 by measuring dissolved gases in continuously stirred cell suspensions of log-phase trophozoites complemented with 10 mM of one of seven substrates: glucose, ethanol, glucosamine, pyruvate, maltose, proline or arginine. Log-phase trophozoites were harvested by centrifugation as described above and washed thoroughly in PBS (x3) immediately prior to each experiment. In order to determine whether individual substrates had an effect on gas production, one-way analysis of variance (ANOVA) and Fisher *a priori* analyses were performed on pooled data using Minitab 13 at a 95% confidence level.

Growth in Bioscreen C. Bioscreen C (Labsystems, Finland), an automated optical density-monitoring system, was used to monitor growth of *S. vortens* in 100 well honeycomb plates. Wells filled with 340 µl of Keister's modified TY-I-S33 culture medium were inoculated with 10 µl of log-phase cultures and turbidity was measured by OD on a vertical light path using a wideband filter (420-580 nm) every 20 min for 120 h. Heat transfer fluid circulation maintained a constant temperature of 25°C and plates were shaken at low amplitude for 5 s before each reading. OD measurements were logged, plotted against time, and exponential growth rates (μ), doubling times (T_d), lag phase length (T_l) and final yields were extrapolated from growth curves by applying the following formula:

$$\mu = 2.303(\log_{10}OD_{t_2} - \log_{10}OD_{t_1}) / (t_2 - t_1), T_d = \ln 2 / \mu$$

$$\text{yield} = OD_{t_f} - OD_{t_0}$$

Growth of the organism was also described by cell counts, using an improved Neubauer haemocytometer. Growth characteristic of this organism are described in detail in Chapter 2. In order to assess the influence of individual substrates on growth, inoculated wells were filled with diluted Keister's modified TY-I-S33 culture medium (final concentration 1:3) and complemented with the following substrates: cysteine, glucose, arginine, proline, serine, maltose, threonine, lysine or leucine. Concentrations were adjusted so that addition of each substrate represented an equivalent carbon input of 60 mM glucose. One-way analysis of variance (ANOVA) was performed on pooled data using Minitab 13 at a 95% confidence level and Fisher *a priori* analyses were used to determine whether individual substrates had an effect on exponential growth rates or final growth yield.

Amino acid analysis of free amino acids in culture medium before and after growth.

Triplicate 15 ml falcon tubes containing 10 ml of Keister's modified TY-I-S33 medium were inoculated with 400 µl of log-phase cultures and incubated at 25°C for 4 to 6 d as specified. Non-inoculated tubes of culture medium were incubated at 25°C for the same amount of time as a control. Following incubation, samples were centrifuged at 800 g for 4 min, and the supernatant was filtered through syringe filters with a porosity of 0.2 µm. Free amino acids in the supernatant were separated from high molecular weight proteins according to the recipe of Pharmacia Biochrom Ltd. (Cambridge, United Kingdom) by mixing 200 µl of 10 %

sulfosalicylic acid with 800 µl of sample, prior to incubation at 4°C for 1 h, and followed by centrifugation at 12,000 g for 10 min. Norleucine (15 nM) was added to the samples as an internal standard, and the amino acid solutions were frozen at -20°C before they were analyzed by ion-exchange chromatography in an amino acid analyzer (Biochrom 30; Pharmacia Biochrom Ltd.). The amino acids were separated by using standard lithium citrate buffers at pH values 2.80, 3.00, 3.15, 3.50, and 3.55. The postcolumn derivatization was performed with ninhydrin, as described by the supplier. Quantification was performed by calibrating the peak heights to an external standard of mixed amino acids at 10 nM (Sigma) as well as by comparison with the internal standard of 15 nM norleucine. The software EZ Chrom Elite (Agilent Technologies) was used to analyze the data. In order to detect significant variations in amino acid concentrations following growth of *S. vortens*, one or 2-sample student t- tests were performed at a 95% confidence level using Minitab 13 (P-values are shown in Appendix 4E).

Amino acid analysis of acid hydrolysate of culture medium before and after growth.

Triplicate 15 ml falcon tubes containing 10 ml of Keister's modified TY-I-S33 medium were inoculated with 400 µl of log-phase cultures and incubated at 25°C for 4 d. Non-inoculated tubes of culture medium were incubated at 25°C for the same amount of time as a control. Following incubation, samples were centrifuged at 800g for 4 min, and supernatant was filtered through 0.2 µM syringe filters. Samples were then placed in soda glass test tubes, and constant boiling HCl was added to a final concentration of 6N. The mixture was stirred under argon for 10 min and tubes were sealed under a constant flow of argon using a butane torch, before being placed in an oven at 110°C for 24 h. Samples were then freeze-dried to remove the acid and resuspended in PBS (pH 6.8). Due to the high cost of this analysis, triplicate samples were then mixed and processed as one sample from that stage. Any remaining proteins were precipitated by addition of 10% sulfosalicylic acid before samples were analysed by ion-exchange chromatography using a Biochrom 30 amino-acid analyser as described above.

Detection of NO production. An iso-NO isolated Nitric Oxide Meter connected to an NO electrode (World Precision Instruments, Florida, USA) was used to detect the production of NO in a washed cell suspension of *S. vortens*. Suspensions of *T. vaginalis* were used as positive controls. The electrode was calibrated by addition of known amounts of NO-saturated buffer. As H₂ was found to induce a strong signal in the NO electrode, a Clark Oxygen electrode was fitted in the reaction vessel to ensure that O₂ levels did not fall below 50 µM, the upper limit of the threshold for H₂ production in *S. vortens*. The temperature was held at 25°C for experiments with *S. vortens* and at 37°C for those with *T. vaginalis*. Stirring was at the constant rate of 150 rev. min⁻¹. Calibrations for O₂ were performed by applying a constant flow of various Air/N₂ mixtures in the headspace above the stirred vortex, using a digital gas mixer (Lundsgaard and Degn, 1973). Solubility for O₂ in PBS was 1.28 mM at 25°C and 0.154 mM at 37°C. Solubility for NO in PBS was 1.94 mM at 25°C and 0.234 mM at 37°C (Wilhelm *et al.*, 1977).

¹H NMR analysis of metabolic products in conditioned buffer. Approximately 8. 10⁷ log-phase trophozoites were harvested as described in above, washed in PBS (pH 6.8), resuspended in 5 ml PBS and incubated at air saturation with or without added glucose (10 mM) for 6 to 10 h as specified. After incubation, the supernatant was filtered through syringe filters with a porosity of 0.2 µm, freeze-dried, then thawed in ²H₂O. ¹H NMR spectra were recorded at 400 MHz on a Bruker DPX400 spectrometer equipped with a 5-mm multinuclear probe and operating at a temperature of 300 K. Spectra were acquired with 90° pulses using an acquisition time of 2.56 s and a relaxation delay of 1 s over a spectral width of 11 ppm over 16 scans. ²H₂O was used as the internal lock and chemical shifts are expressed as parts per million (ppm). Compounds were identified by comparison with spectra of standard solutions.

¹H NMR analysis of metabolic products in suspensions of live cells. Approximately 8. 10⁷ log-phase trophozoites were harvested as previously described, washed in PBS (pH 6.8) and resuspended in 2 ml PBS in a 5 mm NMR glass tube. ¹H NMR spectra of live cells suspensions were recorded at various time intervals in H₂O, using solvent suppression and

capillary $^2\text{H}_2\text{O}$ glass inserts at 400 MHz on a Bruker DPX400 spectrometer equipped with a 5-mm multinuclear probe and operating at a temperature of 300 K. Spectra were acquired with 90° pulses using a pre-saturation period of 5 s and an acquisition time of 2.56 s and a relaxation delay of 1 s over a spectral width of 11 ppm over 128 scans. $^2\text{H}_2\text{O}$ was used as the internal lock and chemical shifts are expressed as parts per million (ppm). Compounds were identified by comparison with spectra of standard solutions.

^{13}C NMR analysis of products of glucose metabolism in suspensions of live cells.

Approximately 10^6 log-phase trophozoites were harvested as previously described, washed in PBS (6.8), and resuspended in 2 ml PBS (6.8) or culture medium as specified, into which 20mM 2- ^{13}C glucose was added. Proton-decoupled ^{13}C NMR spectra were recorded in H_2O with a capillary glass $^2\text{H}_2\text{O}$ insert at 62.9 MHz on a Bruker AV 250 MHz spectrometer equipped with a 5-mm multinuclear probe operating at 300 K. Free induction decay was measured over 32K time domain data points and 256 scans covering a spectral width of 250 ppm with an acquisition time of 2.08 s and a 1 s relaxation delay. $^2\text{H}_2\text{O}$ was used as the internal lock and chemical shifts are expressed as parts per million (ppm). Compounds were first identified using the ^{13}C NMR library of organic compounds of the human metabolic project (<http://www.metabolomics.ca/>) and their identity was confirmed by direct comparison with spectra of standard solutions.

5.4 RESULTS

5.4.1 Influence of substrate on gas metabolism

Rates of CO_2 production, H_2 production and O_2 consumption of washed cell suspensions of *Spironucleus vortens* upon addition of various substrates are summarised in Table 5.1. Of all the substrates tested, only glucose caused a significant increase in both CO_2 and H_2 production (+271% and +302%, respectively; Fisher's *a priori*), as well as O_2 consumption, albeit to a lesser extent (+164%). Addition of ethanol or glucosamine significantly increased CO_2 production, but did not affect H_2 production or O_2 consumption. Pyruvate, maltose or proline had no effect on H_2 production and generated a slight, but non-statistically significant

increases in CO₂ production. Arginine had no effect on H₂ production but its impact on CO₂ production could not be assessed, as addition of this basic compound caused a strong increase in pH (from 6.8 to 8). At this pH, 97.5% of CO₂ produces HCO₃⁻ (pKa HCO₃⁻/CO₂= 6.1), a non-gaseous species, which cannot pass through the silicon membrane, thus rendering mass spectrometric monitoring of CO₂ very insensitive.

Table 5. 1 Influence of substrates on gas metabolism of *Spironucleus vortens*.

Rates of O₂ consumption, CO₂ production and H₂ production were measured by membrane inlet mass spectrometry, upon addition of 10mM substrate in a cell suspension of *S. vortens* within a closed reaction vessel. Significant differences from endogenous rates are determined at a 95% confidence level using Fisher *a priori* analyses. * Indicates lack of data due to pH shifts produced upon addition of arginine.

Substrates	Gas	Gaseous consumption or production rates (nmoles.min ⁻¹ .10 ⁷ cells ⁻¹)	SE	N	Variation from control	Significant difference?
CONTROL No substrate	O ₂	23.50	3.39	3		
	CO ₂	20.30	15.40	3		
	H ₂	19.10	1.03	3		
Glucose	O ₂	62.10	3.89	3	+164%	Yes
	CO ₂	75.30	11.20	3	+271%	Yes
	H ₂	76.80	6.53	4	+302%	Yes
Ethanol	O ₂	33.20	13.10	3	+41%	No
	CO ₂	87.70	35.00	3	+332%	Yes
	H ₂	23.30	10.90	3	+22%	No
Glucosamine	O ₂	24.80	2.43	2	+6%	No
	CO ₂	48.40	6.03	2	+138%	Yes
	H ₂	24.80	5.26	2	+30%	No
Pyruvate	O ₂	24.60	7.76	3	+5%	No
	CO ₂	33.80	9.45	3	+67%	No
	H ₂	20.10	6.87	3	+5%	No
Maltose	O ₂	26.50	6.75	2	+13%	No
	CO ₂	32.60	0.53	2	+61%	No
	H ₂	19.10	6.71	2	0%	No
Proline	O ₂	17.00	1.90	2	-28%	No
	CO ₂	29.70	2.44	2	+46%	No
	H ₂	13.90	1.91	2	-27%	No
Arginine	O ₂	12.60	*	1	-46%	No
	CO ₂	*	*	0	*	*
	H ₂	6.62	*	1	-65%	No

5. 4. 2 Identification of the products of glucose and endogenous metabolism by ^1H NMR

The major compounds released in the supernatant of a cell suspension of *S. vortens* after a 6 h incubation in PBS with 20mM added glucose were identified by ^1H NMR after the supernatant was filtered, freeze-dried and resuspended in $^2\text{H}_2\text{O}$. Duplicate NMR spectra indicated production of acetate, alanine and lactate (Fig. 5. 1). In order to ensure that volatile compounds such as ethanol were not lost in the freeze-drying process, NMR spectra of live cells in H_2O were also analysed using solvent subtraction and $^2\text{H}_2\text{O}$ glass inserts. Results were identical to that of the analysis of freeze-dried supernatant and spectra also showed acetate, alanine and lactate as the only discernable products of metabolism (Fig. 5. 2). Analysis of the supernatant of a cell suspension of *S. vortens* after 6 h incubation in air-saturated PBS, without added glucose revealed production of the same compounds as when cells were incubated with glucose, but in different proportions (Fig. 5. 3). Indeed, when glucose was added, more acetate was produced than alanine, whilst the reverse occurred when no glucose was provided.

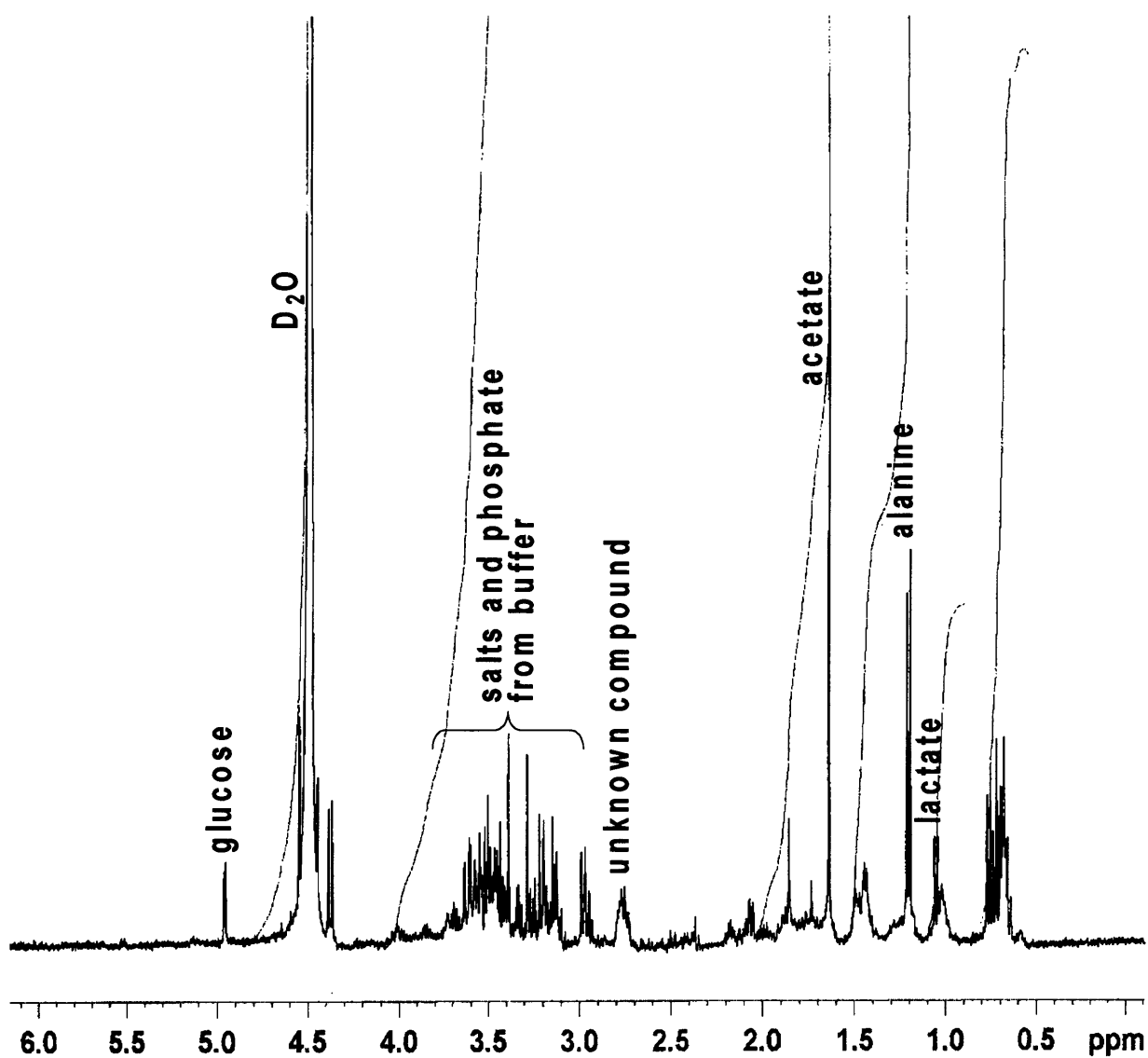


Fig. 5. 1 Representative example of a 400 MHz ^1H $^2\text{H}_2\text{O}$ spectrum showing the products of metabolism in the freeze-dried supernatant of a cell suspension of *Spirochlorella vortens* after incubation in PBS at 25°C for 6h with 20 mM glucose. Duplicate experiments generated identical results (spectrum not shown).

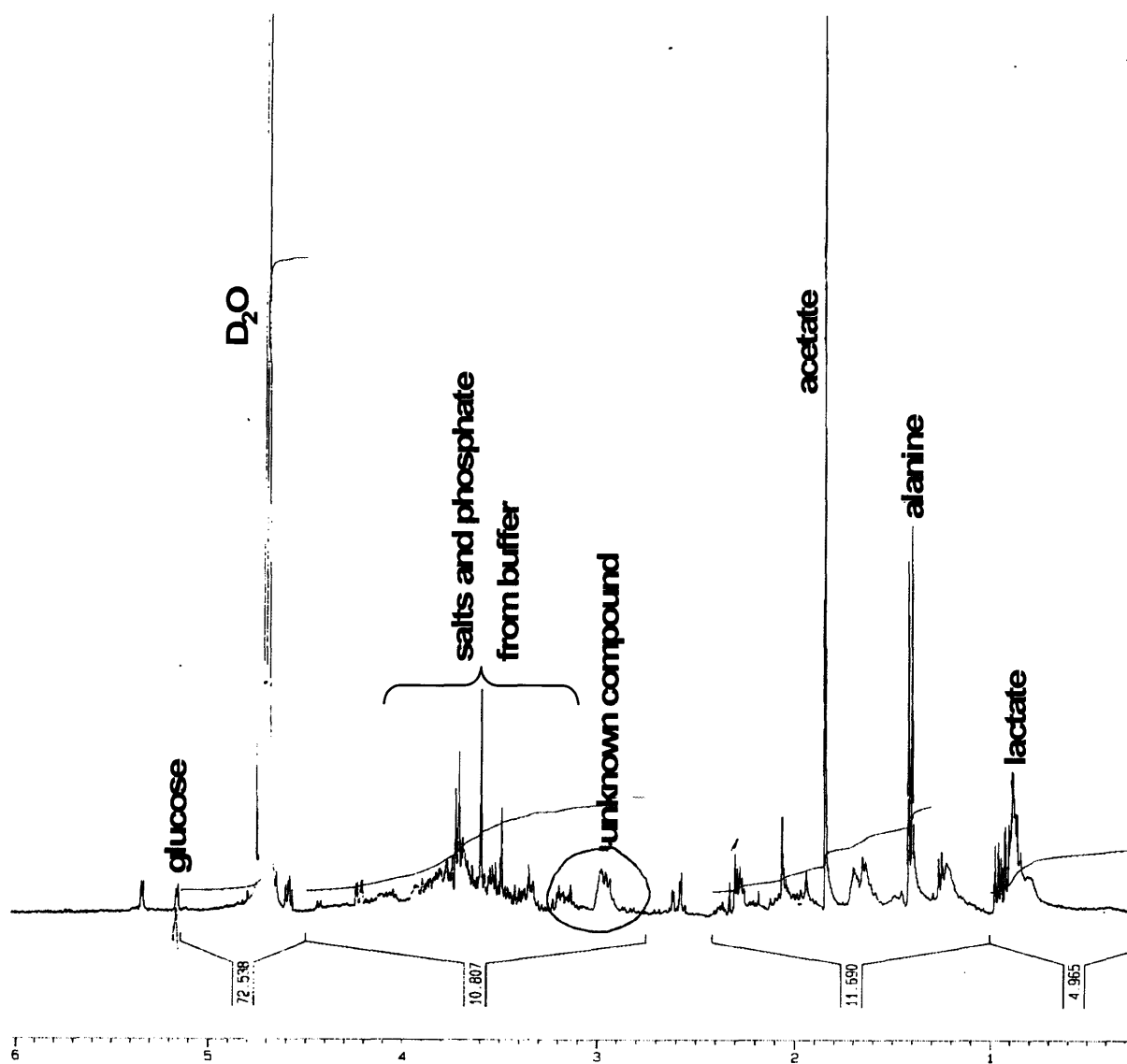


Fig. 5. 2 Representative example of a 400 MHz ^1H H_2O spectrum showing the products of metabolism in a live cell suspension of *Spirochlorella vortens* after incubation in air-saturated PBS at 25°C for 6 h with 20 mM glucose. $^2\text{H}_2\text{O}$ glass inserts were added inside NMR tubes to provide an internal lock. The experiment was performed in triplicate, with identical results (spectra not shown)

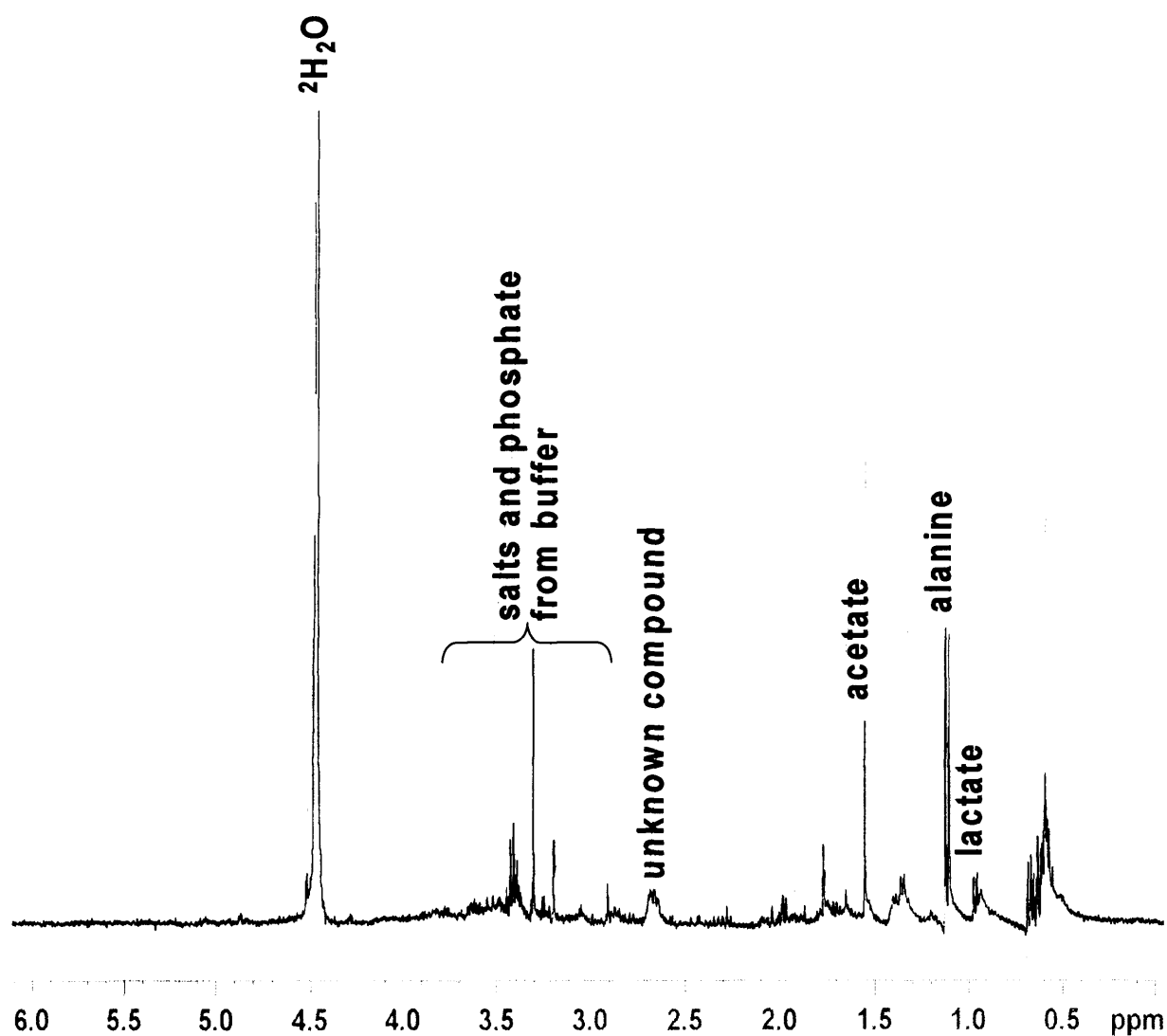


Fig. 5. 3 Representative example of a 400 MHz ^1H $^2\text{H}_2\text{O}$ spectrum showing the products of metabolism in the freeze-dried supernatant of a cell suspension of *Spironucleus vortens* after incubation in air-saturated PBS at 25°C for 6 h without additional glucose. Duplicate experiment generated identical results (spectrum not shown).

5. 4. 3 Identification of the products of glucose metabolism by ^{13}C NMR

The major products of glucose metabolism in live cell suspensions *S. vortens* in buffer with 20 mM added 2- ^{13}C glucose were identified by order of appearance as ethanol, alanine and lactate (Fig 5. 4), in triplicate experiments. Detectable levels of ethanol appeared within 3 h of incubation of 1.52×10^7 cells in 2 ml PBS with 20 mM 2- ^{13}C glucose, after which ethanol

levels rapidly increased, as glucose levels decreased (Fig. 5. 5). When cells were incubated in 1:2 diluted culture medium, with 20 mM added 2-¹³C glucose, detectable levels of ethanol appeared after 15 h.

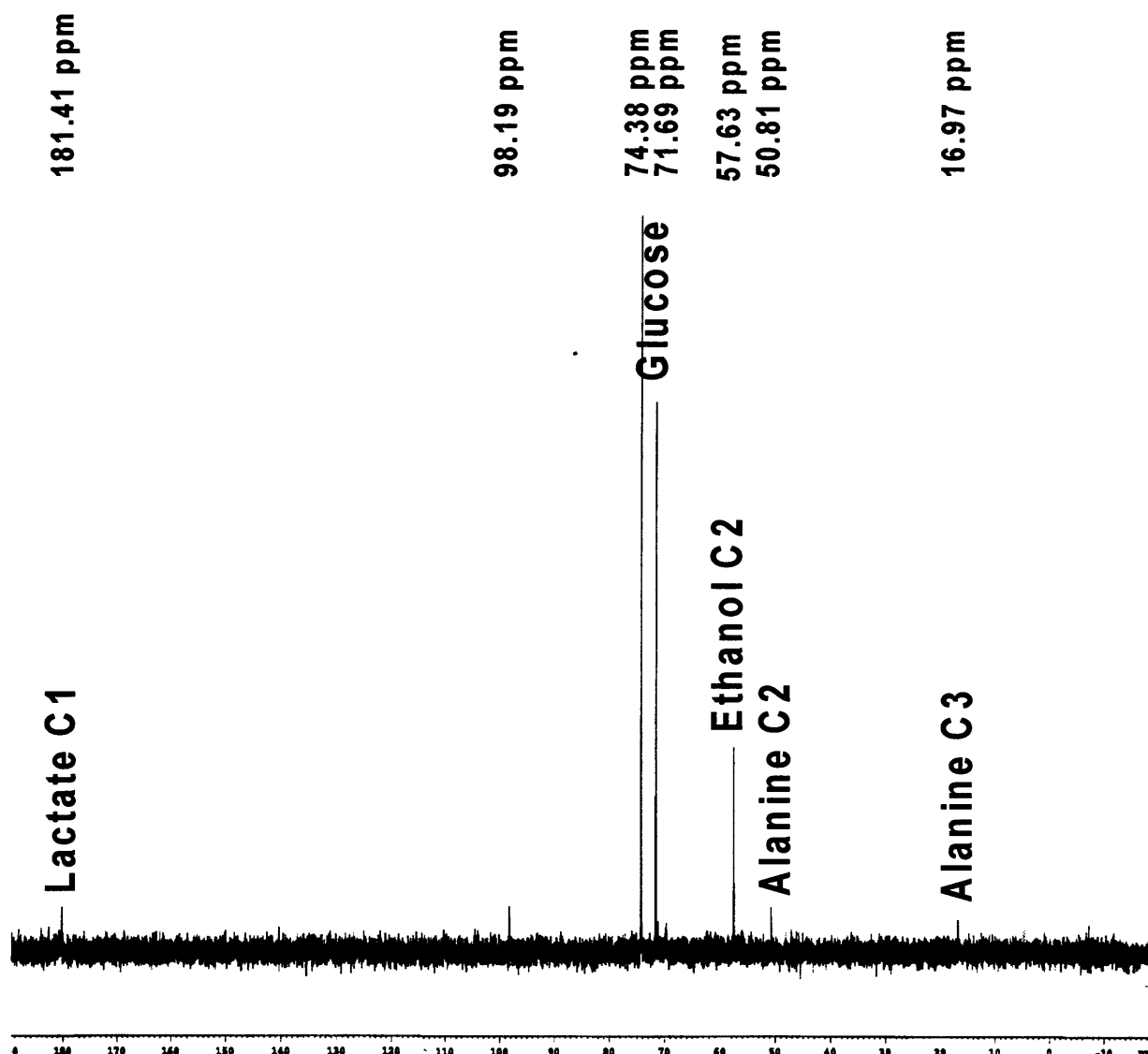


Fig 5. 4. Representative example of a ¹³C NMR spectrum showing the products of glucose metabolism in a live suspension of 2.10×10^6 log-phase *Spirotrunculus vortens*. Trophozoites were incubated in PBS with 2-¹³C labelled glucose for 8 h and spectra were recorded in H₂O at 62.9 MHz on a Bruker AV 250 MHz spectrometer, using ²H₂O glass inserts to provide an internal lock. The experiment was performed in triplicate, with identical results (spectra not shown).

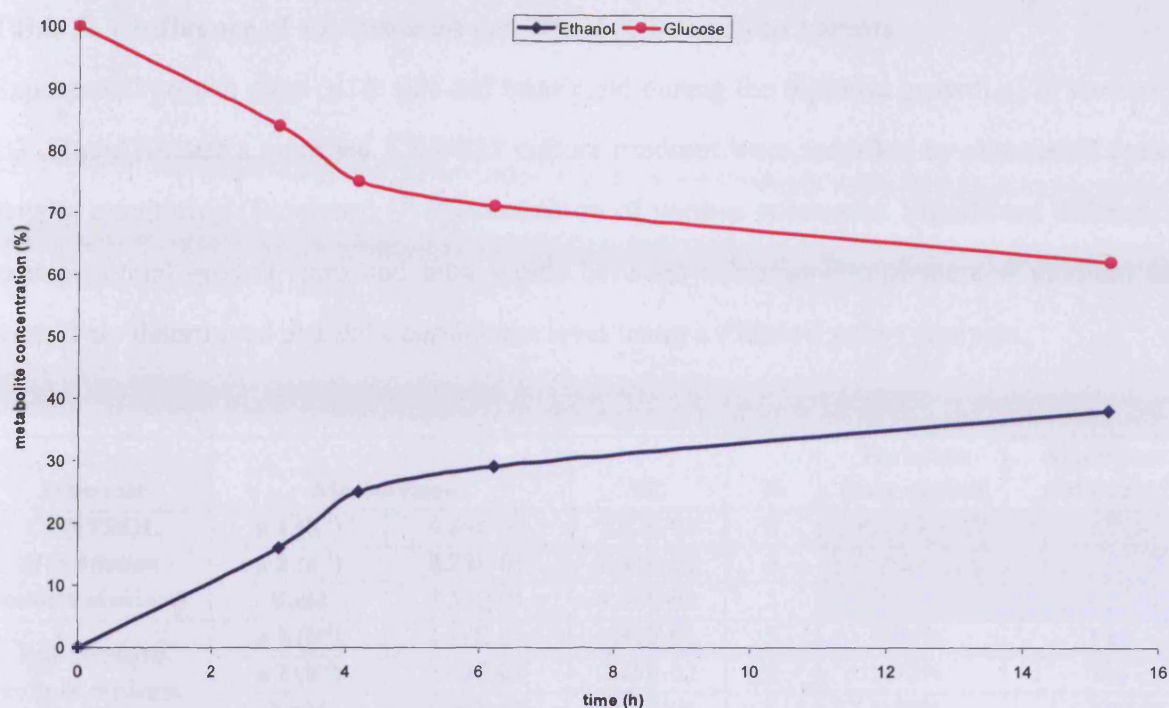


Fig. 5. 5. Kinetic study of 2- ^{13}C glucose catabolism and ethanol production by time Lapse ^{13}C NMR in a suspension of 1.52×10^7 log-phase *Spironucleus vortens* trophozoites in PBS.

5. 4. 4 Influence of substrate on the growth of *S. vortens*

Growth of *S. vortens*, as determined both by automated optical density monitoring (Bioscreen C) and haemocytometer cell counts is fully described in Section 2.2. Briefly, this organism exhibits an atypical biphasic growth curve, and growth can therefore be characterised by the following parameters: exponential growth rate in the first log phase (μ_1), exponential growth rate in the second log phase (μ_2), and the total yield, as indicated by light absorption. The effect of various substrates on the exponential growth rates and the yield of *S. vortens* in 1:3 diluted Keister's modified, TY-I-S33 culture medium, as monitored by Bioscreen C, is summarised in Table 5. 2. Raw bioscreen output is presented in Appendix 5. 4. 4. Of all substrates tested, only glucose significantly increases final growth yield (Fisher's *a posteriori* analysis), but it does not alter growth rates. Arginine, proline, serine and maltose have no effect on growth rates or final yield, while threonine significantly lowers μ_1 , without affecting μ_2 or the total yield. Addition of lysine results in a strong decline in both growth rates and total yield. Addition of leucine completely prevents growth.

Table 5. 2 Influence of substrate on growth of *Spironucleus vortens*.

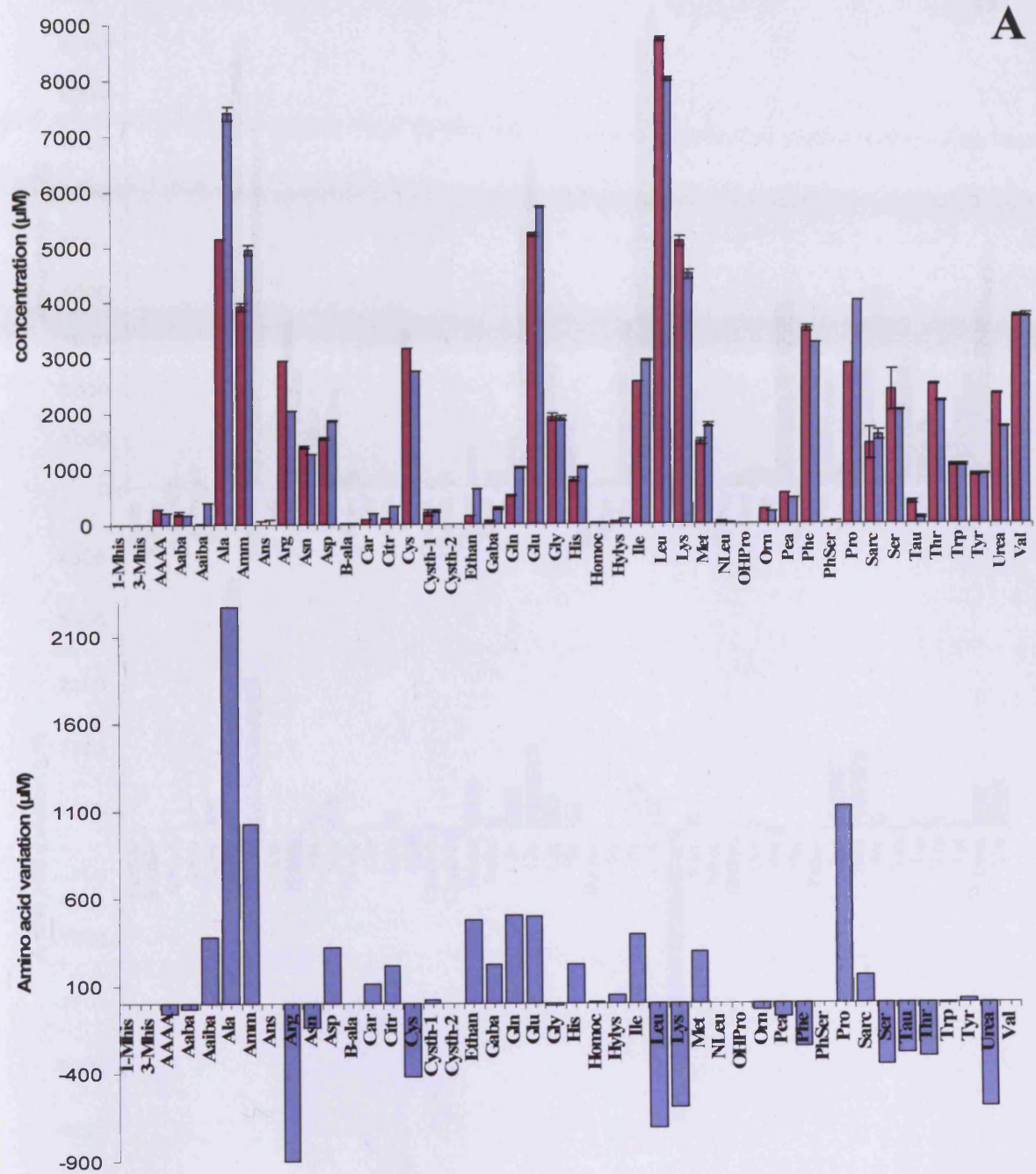
Exponential growth rates (μ_1 & μ_2) and total yield during the biphasic growth of *S. vortens* in 1:3 diluted Keister's modified TY-I-S33 culture medium were recorded by automated optical density monitoring (Bioscreen C) upon addition of various substrates. Significant differences in exponential growth rates and total yields between substrate-complemented medium and control are determined at a 95% confidence level using a Fisher *a priori* analyses.

Substrate	Measurement		SE	N	Variation from control	Significant difference?
CONTROL (1:3 dilution culture medium)	μ_1 (h^{-1})	6.84E-02	2.67E-03	3		
	μ_2 (h^{-1})	2.25E-01	2.31E-02	3		
	Yield	5.55E-01	1.50E-02	3		
Full strength culture medium	μ_1 (h^{-1})	1.19E-01	1.41E-03	3	+74%	Yes
	μ_2 (h^{-1})	3.90E-01	2.15E-02	3	+73%	Yes
	Yield	1.06E+00	3.06E-03	3	+90%	Yes
Glucose 60 mM	μ_1 (h^{-1})	7.20E-02	8.10E-04	2	+5%	No
	μ_2 (h^{-1})	2.19E-01	2.31E-02	2	-3%	No
	Yield	6.01E-01	2.93E-02	2	+8%	Yes
Arginine 60 mM	μ_1 (h^{-1})	6.00E-02	5.58E-04	3	-12%	No
	μ_2 (h^{-1})	1.71E-01	1.12E-02	3	-24%	No
	Yield	5.86E-01	5.86E-01	3	+6%	No
Proline 72 mM	μ_1 (h^{-1})	6.18E-02	2.48E-03	3	-10%	No
	μ_2 (h^{-1})	1.92E-01	4.82E-03	3	-15%	No
	Yield	5.76E-01	1.55E-02	3	+4%	No
Serine 120 mM	μ_1 (h^{-1})	6.38E-02	5.39E-03	3	-7%	No
	μ_2 (h^{-1})	1.86E-01	3.69E-02	3	-18%	No
	Yield	5.71E-01	7.25E-03	3	+3%	No
Maltose 30 mM	μ_1 (h^{-1})	6.21E-02	6.17E-04	3	-9%	No
	μ_2 (h^{-1})	1.82E-01	3.33E-02	3	-19%	No
	Yield	5.56E-01	1.48E-02	3	0%	No
Threonine 90 mM	μ_1 (h^{-1})	4.93E-02	6.74E-03	3	-28%	Yes
	μ_2 (h^{-1})	1.71E-01	1.93E-02	3	-24%	No
	Yield	5.32E-01	9.21E-03	3	-4%	No
Lysine 60 mM	μ_1 (h^{-1})	0.00E+00	0.00E+00	3	-100%	Yes
	μ_2 (h^{-1})	8.29E-02	1.38E-02	3	-63%	Yes
	Yield	7.22E-02	5.81E-03	3	-87%	Yes
Leucine 60 mM	μ_1 (h^{-1})	0.00E+00	*	1	-100%	Yes
	μ_2 (h^{-1})	0.00E+00	*	1	-100%	Yes
	Yield	0.00E+00	*	1	-100%	Yes

5. 4. 5 Amino acid metabolism of *S. vortens*

Analysis of free amino acids concentrations in culture medium before and after growth.

Concentrations of individual free amino acids in culture medium before and after growth of *S. vortens* for 4 to 6 d were determined by ion-exchange flow chromatography and results are summarised in Figure 5. 6. Large amounts of lysine, arginine, leucine, cysteine and urea are being removed from the medium after 4 d of growth (Fig. 5.6A) and. lysine uptake continues to increase as growth processes (Fig. 5. 6B). Smaller but statistically significant (student t-tests, 95% CI) amounts of serine, threonine, taurine, phenylalanine and asparagine are also being metabolised after 4 d of growth (see Appendices 5. 6 A&B). Alanine exhibits the largest increase in concentration after growth, while a large number of other amino acids also demonstrating a significant increase in concentration. Total concentration of free amino acids in fresh culture medium was initially 66 mM, this increased by 3.6 mM and 11 mM after 4 and 6 d growth, respectively (see Appendices 5. 4. 5 A&B).



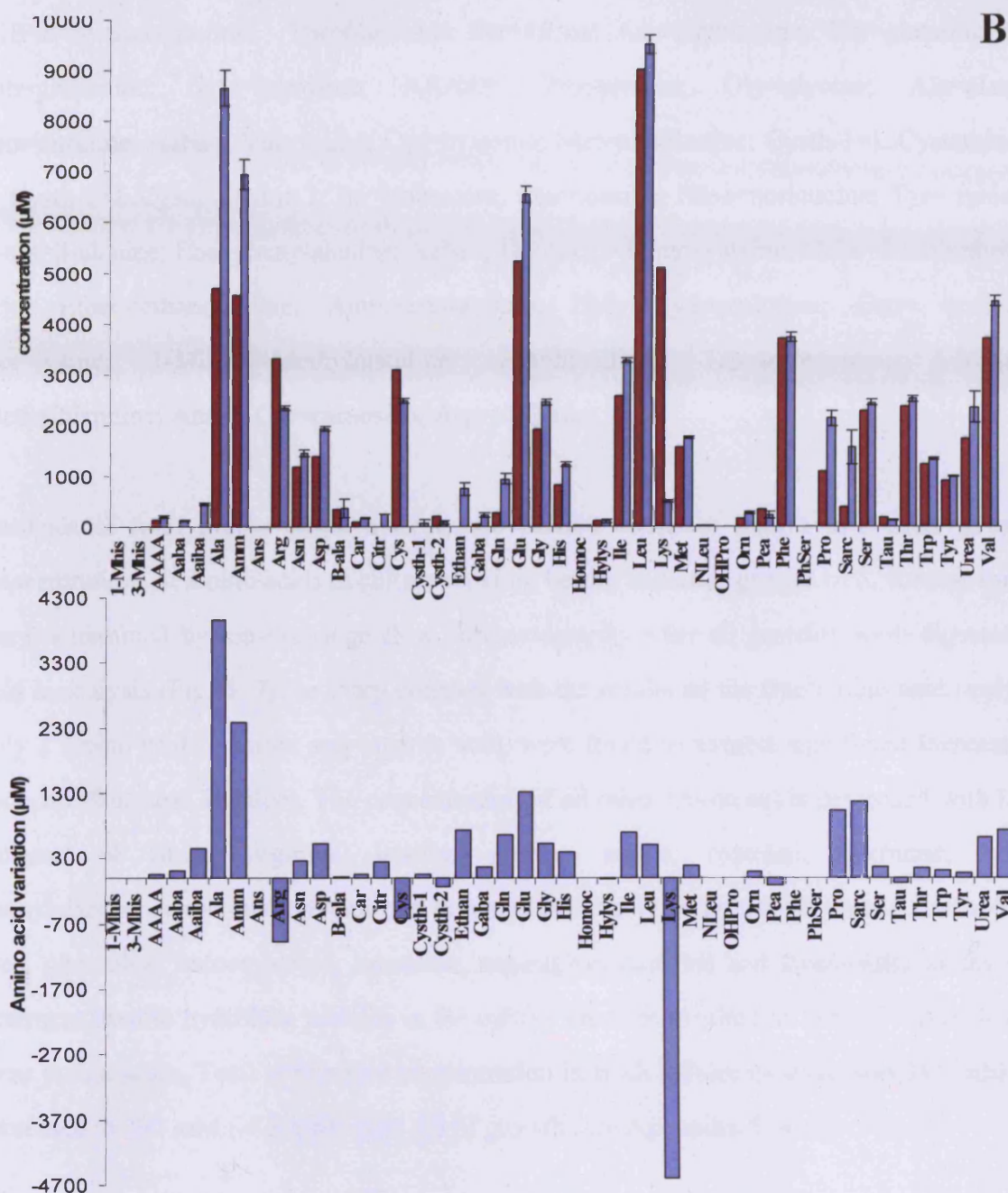


Figure 5. 6 Free amino acids metabolism of *Spironucleus vortens*.

Concentrations of free amino acids in Keister's modified TY-I-S33 culture medium, before (red) and after (blue) growth of *S. vortens* at 25°C for 4 d (A) or 6 d (B), as measured by ion-exchange flow chromatography (biochrom30) are shown on the left panel. Results represent the average of 3 replicates, significant error is plotted around the means. The differences between free amino acid concentrations after and before growth of *S. vortens* are shown on the right panel. Abbreviations: Phser=phenylserine; Tau=taurine; Pea=; Asp=aspartic acid;

OHPro=hydroxyproline; Thr=threonine; Ser=serine; Asn=asparagine; Glu=glutamic acid; Gln=glutamine; Sarc=sarcosine; AAAA=; Pro=proline; Gly=glycine; Ala=alanine; Citr=citruline; Aaba=; Val=valine; Cys=cysteine; Met=methionine; Cysth-1=L.Cystathionine 1; Cysth-2=L.Cystathionine 2; Ile=isoleucine; Leu=leucine; Nleu=norleucine; Tyr= tyrosine; B-ala= β -alanine; Phe=phenylalanine; Aaba=; Homocys=homocysteine; Gaba= δ -aminobutyric acid; Ethan=ethanolamine; Amm=ammonium; Hylys=hydroxylysine; Orn= ornithine; Lys=lysine; 1-Mhis=1-Methylhistidine; His=histidine; Trp=tryptophan; 3-Mhis=3-Methylhistidine; Ans =; Car=carnosine; Arg=arginine

Analysis of total amino acids hydrolysate culture medium before and after growth.

Concentrations of amino acids in culture medium before and after growth of *S. vortens* for 4 d were determined by ion-exchange flow chromatography after all proteins were digested by acid hydrolysis (Fig. 5. 7). In sharp contrast with the results of the free amino-acid analysis, only 2 amino acids, alanine and aspartic acid, were found to exhibit significant increases in concentration (test, P value). The concentration of all other amino acids decreased with large amounts of lysine, arginine, leucine, proline, serine, cysteine, threonine, valine, phenylalanine, ammonium, glycine and glutamic acid being taken up. No data is available for urea, glutamine, homocysteine, carnosine, asparagine, citruline and tryptophan, as the acid treatment used to hydrolyse proteins in the culture medium resulted in total or virtual loss of these amino acids. Total amino acid concentration in fresh culture medium was 297 mM and decreased to 292 mM (-4.8 mM) after 4 d of growth (see Appendix 5. 4. 6).

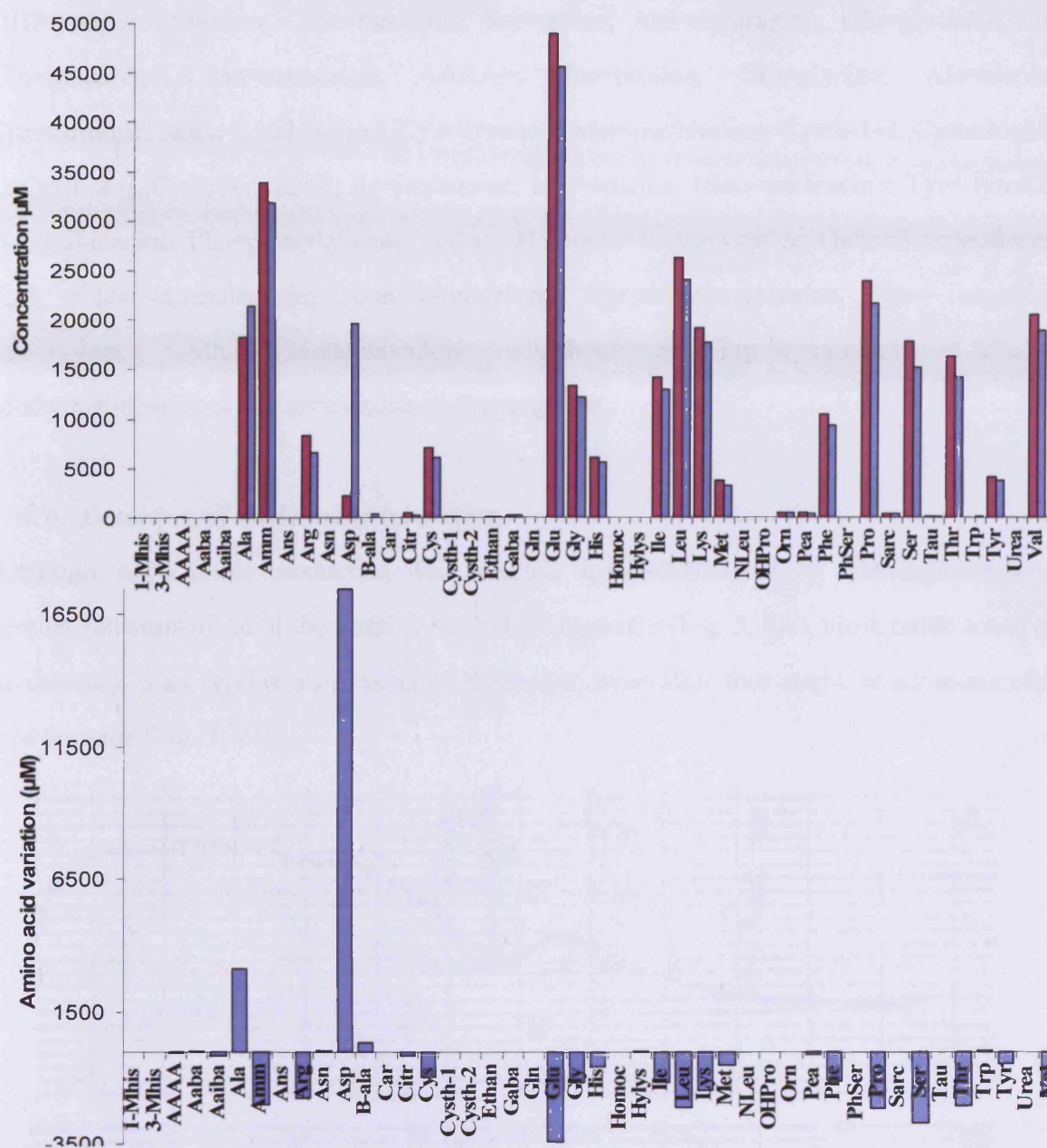


Figure 5. 7 Amino acids metabolism of *Spironucleus vortens*.

Concentrations of amino acids hydrolysate in Keister's modified TY-I-S33 culture medium, before and after growth of *S. vortens* at 25°C for 4 d are shown on the left panel. Proteins were hydrolysed under argon, in 6N constant boiling HCl at 110°C for 24 h and resulting amino acid concentrations were measured by ion-exchange flow chromatography (biochrom30). Results represent the average of 3 replicates. The differences between total amino acid concentrations after and before growth of *S. vortens* are shown on the right panel.

Abbreviations: Phser=phenylserine; Tau=taurine; Pea=; Asp=aspartic acid;

OHPro=hydroxyproline; Thr=threonine; Ser=serine; Asn=asparagine; Glu=glutamic acid; Gln=glutamine; Sarc=sarcosine; AAAA=; Pro=proline; Gly=glycine; Ala=alanine; Citr=citruline; Aaba=; Val=valine; Cys=cysteine; Met=methionine; Cyth-1=L.Cystathionine 1; Cyth-2=L.Cystathionine 2; Ile=isoleucine; Leu=leucine; Nleu=norleucine; Tyr= tyrosine; B-ala= β -alanine; Phe=phenylalanine; Aaba=; Homocys=homocysteine; Gaba= δ -aminobutyric acid; Ethan=ethanolamine; Amm=ammonium; Hylys=hydroxylysine; Orn= ornithine; Lys=lysine; 1-Mhis=1-Methylhistidine; His=histidine; Trp=tryptophan; 3-Mhis=3-Methylhistidine; Ans =; Car=carnosine; Arg=arginine

5. 4. 6 . Detection of nitric oxide formation

Although, nitric oxide production was detected upon addition of 20 mM arginine in an aerobic cell suspension of the positive control, *T. vaginalis* (Fig. 5. 8A), nitric oxide could not be detected in an aerobic suspension of *S. vortens*, even after four stepwise additions of 20 mM arginine (Fig. 5. 8 B)

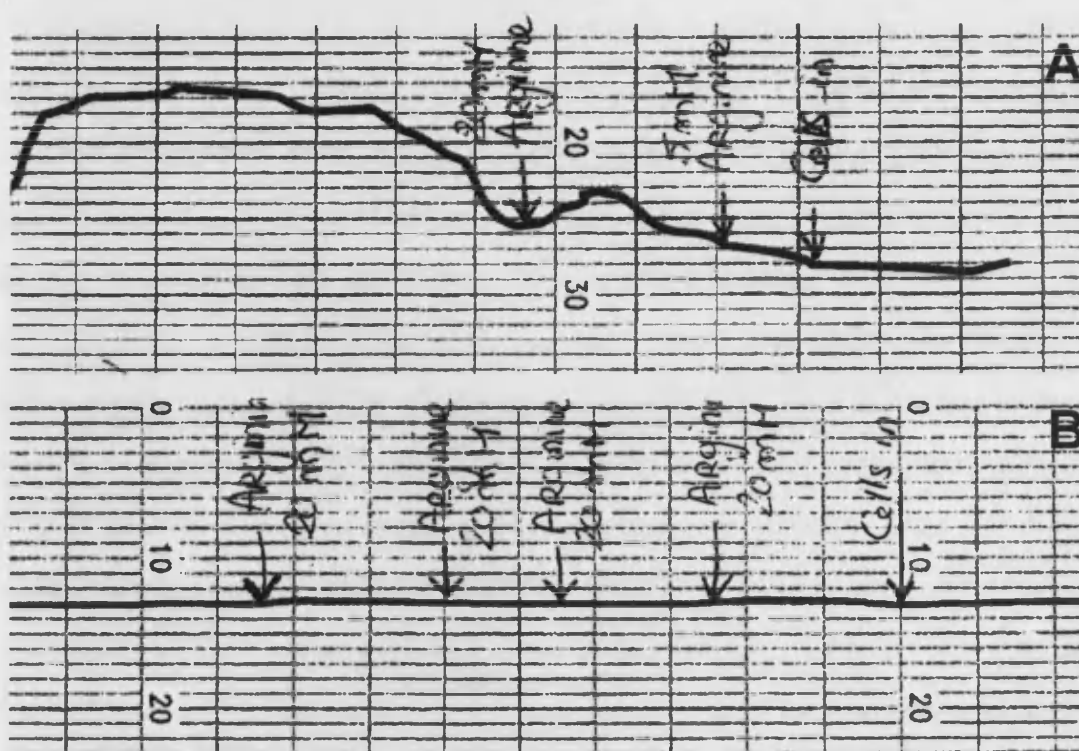


Fig. 5. 8 NO detection in washed, aerobic suspensions of *Trichomonas vaginalis* and *Spironucleus vortens* (B) upon addition of arginine, as recorded by an iso-NO isolated Nitric Oxide Meter connected to an NO electrode (World Precision Instruments, Florida, USA).

5. 5 DISCUSSION

Mass spectrometric analysis of gas metabolism and automated density monitoring of growth reveal that glucose is the only substrate to have a positive impact on both the gas metabolism (Table 5. 1) and growth (Table 5. 2) of *Spironucleus vortens*. Glucose significantly increases both H₂ and CO₂ production rates, as well as O₂ consumption in cell suspensions, and its addition to diluted culture medium increases the final growth yield by 8%. Interestingly, although glucose enhances the final yield, its addition does not alter the initial exponential growth rates of the organism, which suggests that it is not the substrate of choice for this organism but that it is only used once the primary substrate is depleted from the culture medium.

If glucose is only an alternative energy source, the identity of the primary fuel compound for this organism remains unknown. Several of the most likely alternative energy-yielding molecules, such as maltose, the main energetic molecule in *T. vaginalis* (ter Kuile 1994a&b; Petrin, Delgaty et al. 1998), arginine, which is used as an alternative carbohydrate energy source by both *T. vaginalis* and *Giardia intestinalis* (Linstead and Cranshaw 1983; Schofield, Costello et al. 1990; Schofield and Edwards 1991; Schofield, Edwards et al. 1992; Petrin, Delgaty et al. 1998), and proline, the main substrate of *Trypanosoma brucei* while it infects its insect host (Evans and Brown 1972; Ford and Bowman 1973; Krassner, Sylvester et al. 1973; Sylvester and Krassner 1976; Lamour, Riviere et al. 2005), were shown to have no effect on either gas metabolism or growth of *S. vortens*. Pyruvate, serine and threonine also had no effect, while surprisingly, lysine and leucine caused a strong inhibition in growth of *S. vortens*. The concentration of lysine and leucine that was added to cells (60 mM) in the growth monitoring experiment was chosen to generate an equivalent carbon input to that of 60 mM of glucose, the concentration that is normally present in the growth medium. This concentration of lysine and leucine is, however, much higher than what would normally be found in culture medium (19 and 26 mM, respectively, see Appendix 5.8), which might explain the observed toxicity to the organism. Future work should include monitoring of growth, but using lower concentrations of the above substrates..

The status of glucose as an alternative energy source for *S. vortens* was also supported by ^{13}C NMR data. Indeed, when 2- ^{13}C glucose was added to a washed cell suspension in buffer, it was metabolised after 3 h (Fig. 5. 5), but when added to cells in culture medium, it was only metabolised to detectable levels after 15 h, which implies that *S. vortens* only metabolises the compound as a last resort.

It should be noted that mass spectrometry indicates that both ethanol and glucosamine induce large increases in CO_2 production rates by *S. vortens*, but that these substrates do not influence H_2 production. This suggests that neither compound is used in the ATP-generating pathway of glucose fermentation, as hypothesized in Chapter 4 (see Fig. 4. 4), which would result in the formation of one molecule of H_2 by molecule of oxidised glucose. In turn, this infers that alternative substrate-level, energy-generating pathways might be available.

Products of *S. vortens* metabolism upon addition of glucose were identified by ^1H NMR as acetate, alanine and lactate (Fig. 5. 1). Production of lactate is also found in fermentative metabolism of the free-living relative *Hexamita inflata* (Biagini, McIntyre et al. 1998), but interestingly is absent in the closest parasitic relative, *Giardia intestinalis* (see Lindmark, 1980; Weinbach et al. 1980; Jarroll et al. 1981). The lack of ethanol detection by ^1H NMR in conditioned supernatant is intriguing, as the compound is a major product of fermentative metabolism in both *H. inflata* and *G. intestinalis* (Lindmark 1980; Weinbach, Claggett et al. 1980; Jarroll, Manning et al. 1989; Lloyd and Paget 1991; Schofield and Edwards 1991; Biagini, McIntyre et al. 1998), and it was detected as a product of 2- ^{13}C glucose metabolism of *S. vortens* by ^{13}C NMR (Fig. 5. 4). ^1H NMR analysis of live cells suspensions in H_2O , using solvent suppression and $^2\text{H}_2\text{O}$ inserts as internal locks, also failed to detect ethanol. These observations eliminate the possibility that this volatile compound was lost during the freeze-drying stage of supernatant analysis by ^1H NMR. It is possible that ethanol is only produced at certain growth stages. Indeed, log-phase trophozoites were used in this experiment, and some organisms, such as *G. intestinalis* have been shown not to produce ethanol until the later stages of growth (Lindmark 1980; Schofield and Edwards 1991). Alternatively, ethanol might have been further metabolised by the organism, as it was observed that, aside from being a product of glucose metabolism (Fig. 5. 4), this compound could be utilised as a substrate by the organism, resulting in increased CO_2 but not H_2

production rates. This behaviour is well characterised in other eukaryotic microorganisms, such as *Saccharomyces* species, which accumulate ethanol during the first part of diauxic growth, then start metabolising it to acetate, with a concurrent increase in growth rate, thus exhibiting a typical biphasic growth (Haarasilta and Oura 1975; Toda and Yabe 1979; Toda, Yabe et al. 1980; Ramakrishnan and Hartley 1993). This would be consistent with the observed biphasic growth pattern of *Spironucleus vortens* (see Chapter 2, Fig. 2.1), the fact that experiments were conducted at high O₂ tensions (in buffer with a large headspace and without reducing agents) and the detection of large amounts of acetate in conditioned buffer by ¹H NMR. The much lower cell numbers used in the ¹³C NMR experiments (on average 2.5x10⁶ cells/ml as opposed to 3x10⁷ cells/ml in ¹H NMR experiments) could explain why ethanol can still be detected and acetate is not produced in these experiments. It is also possible that acetate production does not result from glucose metabolism, but comes from a different pathway, as it is the case in *G. intestinalis* (Schofield and Edwards 1991), however the glucose-dependant increase of acetate production observed by ¹H NMR would tend to invalidate that hypothesis. In order to investigate these hypotheses, future work should include repeat the ¹H NMR and ¹³C NMR experiments with a lower cell count, using a more sensitive spectrometer and cells at various growth stages.

S. vortens trophozoites were shown to survive and remain actively swimming for over 10 h in buffer without added substrates (see Chapter 2), which infers that they possess large stores of endogenous substrates. ¹H NMR analysis reveals that the products of such endogenous metabolism; namely acetate, alanine and lactate; are identical to those of metabolism when glucose is provided, but that they occur in different proportions. Indeed, alanine production exceeds that of acetate when no glucose is provided (Fig. 5. 3), while the reverse is true in the presence of glucose (Fig. 5. 1). As alanine is typically produced when amino groups are transferred from glutamate to pyruvate during amino acid catabolism (Lockwood and Coombs 1991a; Nelson and Cox 2000; Melhorn 2009), production of larger amounts of alanine during endogenous metabolism suggests that storage molecules are likely to consist largely of amino acids or proteins.

Amino acid analysis of conditioned growth medium also shows that large quantities of alanine, along with aspartic acid, are produced by *S. vortens*. This implies that catabolism of

exogenous amino acids might occur during growth in culture medium. Ammonia assimilation probably also contributes to alanine production. Indeed, approximately 2 mM of ammonia is taken up after 4 d of growth (Fig. 5. 7) and formation of alanine and aspartate during the assimilation of ammonia, is a well-documented occurrence in many bacteria (Rigano, Aliotta et al. 1975; Rowell and Stewart 1975; Johansson and Gest 1976; Aharonowitz and Friedrich 1980; Kenealy, Thompson et al. 1982). Additionally, the uptake of amino acids by *S. vortens* appears too high to be justified solely by amino acid auxotrophy, which further hints at the use of amino acids as energy-yielding molecules. This appears especially likely, as glucose does not seem to be the first choice for an energy-generating molecule. However, all these findings are somewhat at odds with the lack of metabolic or growth stimulation (Tables 5. 1 & 5. 2) upon addition of those amino acids, which are taken up in the largest proportions during growth (Figs. 5. 6 & 5. 7). A specific example of this inconsistency is arginine. Indeed, up to 1.8 mM of this compound is taken up by the organism after 4 d of growth. As arginine is used to generate energy by substrate-level phosphorylation, with formation of ornithine and nitric oxide, via the arginine dihydrolase pathway in *T. vaginalis* and *G. intestinalis* (Yarlett, Martinez et al. 1996; Yarlett, Martinez et al. 2000; Harris, Goldberg et al. 2006), the utilisation of arginine as a fuel by *S. vortens* would seem likely. However, the compound does not stimulate the gas metabolism or the growth of *S. vortens*, suggesting that it is not used as an energy-generating substrate. Moreover, addition of arginine to a cell suspension does not induce nitric oxide formation (Fig. 5. 8), and no significant ornithine production was detected by ion exchange chromatography, which rules out the presence of a significant energy-generating arginine dihydrolase pathway in this organism.

Analysis of free amino acid concentrations in conditioned culture medium, without protein hydrolysis, recorded that the concentration of a large number of amino acids increased in culture medium during growth of the organism. The total concentration of free amino acids increased by 3.6 mM after 4 d growth and 11 mM after 6 d growth. This finding is in complete contrast with the result of total amino acid analysis with protein hydrolysis, in which only alanine and aspartate levels were found to increase, and the total concentration of amino acids decreased by 4.8 mM after 4 d growth. This discrepancy suggests that *S. vortens* hydrolyses large amounts of proteins from the culture medium, presumably through the action

of extracellular proteases. Such a behaviour is intriguing, as the concentration of free amino acids within the culture medium (66 mM, see Appendix 5. 4. 5) is more than sufficient to cater for the amount (4.8 mM) consumed by the parasite. Additionally, comparison between the uptake of individual amino acids and their original levels shows that no individual amino acid acts as a limiting substrate. Indeed, no single amino acid is seen to be completely used up in culture medium after 4 days of growth. Such high levels of protein hydrolysis therefore appear unnecessary *in vitro*, however, they might be directly relevant within the host, not only to scavenge nutrients but also to increase virulence, as a means of crossing the gut barrier and establishing systemic infections (North 1991a&b; Petrin, Delgaty et al. 1998).

This study clearly demonstrates that metabolism of *S. vortens* is significantly different from that of its closest free-living or parasitic relatives. Much remains to be elucidated about the unique metabolism of this pathogen, and future work should include mass spectrometric and bioscreen investigations of more potential substrates, as well as the use of ¹³C-labelled compounds to determine the fate of the large quantities of amino acids consumed by this organism during growth.

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CHAPTER 6: THE DIPLOMONAD PARASITE *SPIRONUCLEUS VORTENS* HAS BOTH A HIGH AFFINITY AND A HIGH TOLERANCE FOR O₂

6.1 ABSTRACT

Spiroucleus vortens, a diplomonad flagellate parasite of fish, and reported to lack redox active organelles, was described as an aerotolerant anaerobe, however, no biochemical investigation of its actual affinity for oxygen has yet been conducted. In this study, oxygen affinity (K_m) and inhibition threshold (K_t) were investigated using a Clark oxygen electrode in an open system and the effects of varying oxygen tensions on the endproducts of glucose metabolism were recorded by ¹³C NMR. Because of the temperature at which O₂ electrode experiments were conducted, gas exchange rates were low, and experimental design was thus highly challenging. Nevertheless, O₂ electrode experiments revealed that the organism was unusual in that it combined a very high affinity for O₂ ($K_m=0.104\ \mu\text{M}$) with a tolerance for high concentrations of the gas ($K_t=65.14\ \mu\text{M}$). In addition, ¹³C NMR analysis of glucose metabolism demonstrated that variations in concentrations of dissolved O₂ affected the nature and proportion of metabolic endproducts: under complete anaerobiosis, lactate, ethanol and alanine were produced, while under 2% and 5% O₂, only lactate and ethanol were detected, with the ratio of ethanol to lactate increasing with O₂ content.

6.2 INTRODUCTION

Spironucleus vortens is a small, eukaryotic flagellate, which has been described as lacking both mitochondria and hydrogenosomes (Brugerolle, Joyon et al. 1973; Brugerolle 1975; Cavalier-Smith 1987; Poynton, Fraser et al. 1995), and was classified as an aero-tolerant anaerobe (Poynton, Fraser et al. 1995). While some authors initially regarded its lack of mitochondria as the primitive attribute of a basal eukaryote (Cavalier-Smith 1987), many now speculate that it might be an evolutionary adaptation of a primarily aerobic organism to the anaerobic lifestyle (Embley and Hirt 1998; Lloyd and Harris 2002; Embley and Martin 2006).

Other amitochondriated flagellates, such as *Giardia intestinalis* and *Hexamita inflata*, were also originally described as anaerobes or aero-tolerant anaerobes, but were later discovered to benefit from the presence of low levels of O₂ in their growth medium, and were therefore reclassified as microaerophiles (Paget, Lloyd et al. 1989; Paget, Manning et al. 1993; Biagini, Suller et al. 1997; Biagini, Park et al. 2001). The same is true of *Trichomonas vaginalis* (Paget and Lloyd 1990), which lacks fully functional mitochondria, but instead possesses an “anaerobic” type of redox-active organelle, the hydrogenosome (Lindmark and Muller 1973; Johnson, Lahti et al. 1993), and in which the doubling time can be drastically reduced from 5 to 2.2 h upon addition of small traces of O₂ (Paget and Lloyd 1990). All these organisms have been shown to favour low O₂ concentrations *in vitro*, but their ecological niches are characterised by the presence of low and fluctuating levels of O₂ and they have therefore evolved efficient O₂ scavenging and detoxification systems (Lloyd and Pedersen 1985; Ellis, Wingfield et al. 1993; Paget, Manning et al. 1993; Williams and Lloyd 1993; Ellis, Yarlett et al. 1994; Tekwani and Mehlotra 1999; Lloyd, Harris et al. 2000; Biagini, Park et al. 2001; Rasoloson, Tomkova et al. 2001; Di Matteo, Scandurra et al. 2008). Small variations in O₂ tensions were shown to have a strong influence, not just on the growth rate, but also on the carbon balance of these organisms, as the nature and relative proportions of metabolic end-products vary with increasing O₂ tensions (Paget, Raynor et al. 1990; Lloyd and Paget 1991; Ellis, Cole et al. 1992; Paget, Kelly et al. 1993). Intracellular oxygen tensions are also very important in chemotherapy of “anaerobic” protists, as decreased activity in O₂

scavenging by an NADH oxydase in *G. intestinalis* and *T. vaginalis*, was shown to generate a degree of resistance to metronidazole, by decreasing the rate of activation of the drug (Gillin and Reiner 1982; Lloyd and Pedersen 1985; Yarlett, Yarlett et al. 1986; Ellis, Cole et al. 1992; Ellis, Wingfield et al. 1993; Ellis, Yarlett et al. 1994; Rasoloson, Tomkova et al. 2001).

S. vortens is primarily a gut commensal, but it is known to colonise the liver, kidneys and epithelium of systemically infected cichlids (Poynton, Fraser et al. 1995; Paull and Matthews 2001), which suggests that the organism may have the ability to cope with much larger variations in O₂ concentration than the trophozoites of *G. intestinalis* or *T. vaginalis*. *In vitro*, it also exhibits surprising levels of oxygen tolerance, as it can grow in tubes with a large headspace of air, and was shown to survive in air saturated-buffer for over 10 h (see Chapter 2). However, the actual affinity for oxygen (K_m), as well as the threshold for oxygen inhibition (K_t) in this organism has not yet been established. Similarly, the effect of dissolved O₂ concentration on the flux of carbon end products, which has been extensively described in *G. intestinalis* (Paget, Kelly et al. 1993), *H. inflata* (Biagini, McIntyre et al. 1998) and *T. vaginalis* (Ellis, Cole et al. 1992), has not been described in this organism.

In this study, K_m and K_t for O₂ in *S. vortens* were investigated using an open, O₂ electrode set up. This was made challenging by the low temperature at which experiments were conducted (25°C), as gas exchanges at this temperature are extremely slow, thus requiring for trophozoites to be stirred vigorously in buffer for several hours. The effects of varying concentrations in dissolved O₂ on carbohydrate metabolism were also analysed by ¹³C nuclear magnetic resonance (NMR).

6.3 MATERIAL AND METHODS

Organisms and Cultures. *Spironucleus vortens*, ATCC 50386, were grown in TY-I-S33 medium as described in Chapter 2. Organisms in their exponential phase of growth were harvested by centrifugation at 800 g for 2 min at room temperature in a bench centrifuge (MSE minor).

Determination of O₂ affinity and inhibition threshold. A stirred, 6ml stainless steel reaction vessel (Degn et al, 1980) fitted with a Clark oxygen electrode (Radiometer, Copenhagen) was filled with 3.5 ml of PBS. Temperature was held at 25°C by circulating thermostatically maintained water around the reaction vessel and stirring was at the constant rate of 150 rev. min⁻¹. Calibrations were performed by applying a constant flow of various 5% O₂/N₂ or air/N₂ mixtures in the headspace above the stirred vortex, using a digital gas mixer (Lundsgaard and Degn, 1973). Half-time for saturation ($t_{1/2}$) of N₂-saturated buffer with 5% O₂ was also determined. Approximately 10⁶ log-phased trophozoites were then harvested, washed (x3) in PBS (pH=6.8) as described above, resuspended in 3.5 ml of PBS and introduced into the reaction vessel. Steady state levels of dissolved O₂ in cell suspensions (T_L) during 10 stepwise increases in applied O₂ tensions (T_G) were measured and respiratory rates (V_R) were calculated according to the following formula:

$$V_R = \ln 2 * (T_G - T_L) / t_{1/2}.$$

Experiments were performed, for O₂ tensions ranging from 0 to 20.91% (2 replicates) and from 0 to 5% (2 replicates), using respectively air/N₂ or 5%O₂/N₂ gas mixtures. O₂ affinity constant (K_m) and O₂ inhibition threshold (K_t) were extrapolated from the double reciprocal plot $1/V_R$ against $1/T_L$ (Fig 6.1). Solubility for oxygen in PBS buffer at 25°C was approximated to 1.28 mM (Wilhelm, Battino *et al.*, 1977).

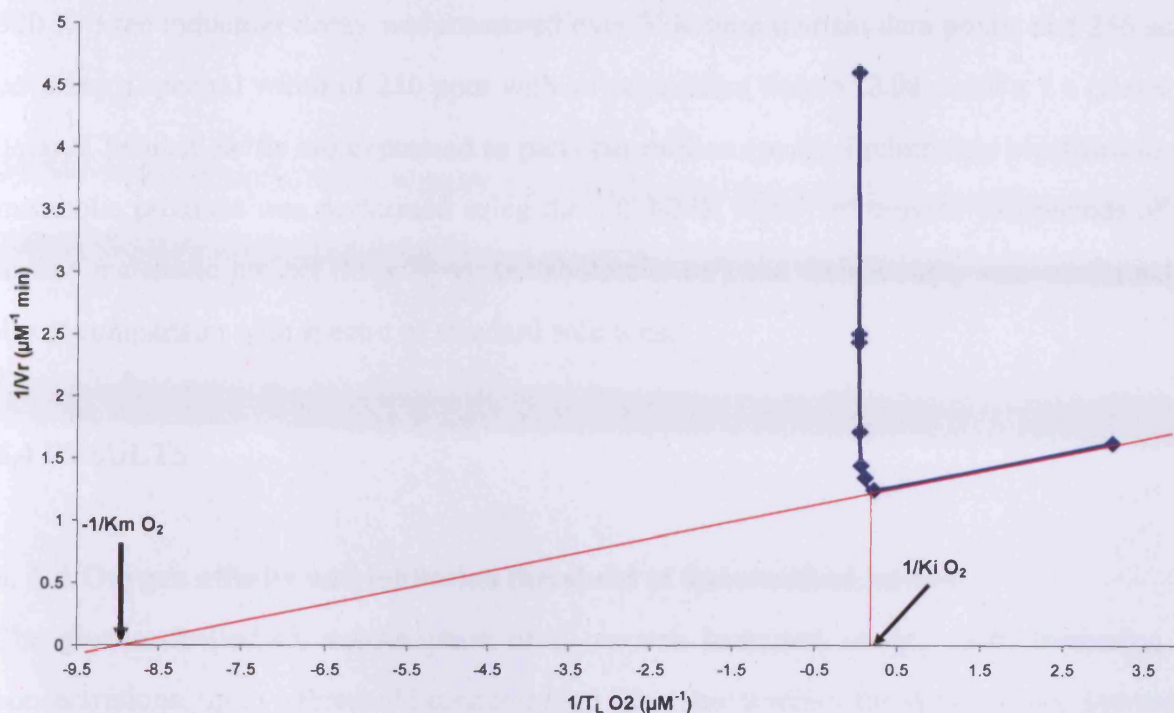


Fig 6. 1. Double reciprocal plot of O_2 consumption against increasing O_2 tension (0 to 5% or 0 to 20.9% O_2) for a suspension of *Spironucleus vortens* (2.45×10^5 cells. ml^{-1}), to which 10mM glucose was added. The intercept of the positive slope for the curve with the X axis corresponds to the inverse value of the oxygen affinity (K_m) for O_2 and the X coordinate of the point with the lowest Y value indicates the threshold for O_2 inhibition (K_t).

^{13}C NMR analysis of products of glucose metabolism under various O_2 tensions.

Approximately 10^6 log-phased trophozoites were harvested as previously described, washed and resuspended in PBS (pH 6.8), and incubated in a fermenter with 20mM 2- ^{13}C glucose for 12h. Temperature was held at 20°C by circulating thermostatically maintained water around the fermenter and stirring was at the constant rate of $100 \text{ rev. min}^{-1}$. Various O_2 tensions were maintained by applying a constant flow of 5% O_2/N_2 mixtures in the headspace above the stirred vortex, using a digital gas mixer, as described above. Following incubation, viability of cells was investigated by examining their motility under a light microscope and supernatant was recovered and filtered through a $0.22 \mu\text{M}$ syringe filter. $^2\text{H}_2\text{O}$ (10% v/v) was added as an internal lock. Proton-decoupled ^{13}C NMR spectra were then recorded in H_2O at 62.9 MHz on a Bruker AV 250 MHz spectrometer equipped with a 5-mm multinuclear probe operating at

300 K. Free induction decay was measured over 32K time domain data points and 256 scans covering a spectral width of 250 ppm with an acquisition time of 2.08 s and a 1 s relaxation delay. Chemical shifts are expressed as parts per million (ppm). Preliminary identification of metabolic products was performed using the ^{13}C NMR library of organic compounds of the human metabolic project (<http://www.metabolomics.ca/>) and their identity was confirmed by direct comparison with spectra of standard solutions.

6.4 RESULTS

6.4.1 Oxygen affinity and inhibition threshold of *Spironucleus vortens*

The glucose-fuelled O_2 consumption of *S. vortens* increased linearly with increasing O_2 concentrations, up to a threshold concentration (K_t), above which the uptake of O_2 decreased. For experiments using air/ N_2 mixtures, in which O_2 increased from 0 to 20.91% by 2.09% steps, the apparent oxygen affinity constant (K_m) equalled $1.207\ \mu\text{M}$ ($n=2$, $\mu\text{C}[0.487;1.927]$) and $K_t=65.15\ \mu\text{M}$ ($n=2$, $\mu\text{C}[35.71; 92.59]$). For experiments using 5% O_2/N_2 mixtures, in which O_2 increased from 0 to 5% by 0.5% steps, $K_m=0.104\ \mu\text{M}$ ($n=2$, $\mu\text{C}[0.099; 0.109]$) and $K_t=6.78\ \mu\text{M}$ ($n=2$, $\mu\text{C}[5.75; 7.81]$). The average K_m for all experiments was $0.66\ \mu\text{M}$ ($\pm 0.43\ \mu\text{M}$, $n=4$) and K_t was $35.47\ \mu\text{M}$ ($\pm 20.23\ \mu\text{M}$, $n=4$). As gas exchanges were slow at this temperature, completion of the 10 stepwise increases in O_2 concentrations for both types of experiments took over 4h. By the end of the experiment, trophozoites appeared non viable, as indicated by their lost of motility, observed by light microscopy. Because *S. vortens* was highly efficient at scavenging O_2 , T_I in the first two stepwise increases in O_2 concentration was equal to 0. As a result, $1/T_I$ was infinite and only 2 points were available to calculate the equation of the tangent that permitted to derive the value of K_m . Interpretation of this experiments should therefore take into account the lack of reliability in the determination of K_m .

6.4.2 Products of glucose metabolism under various O_2 tensions (^{13}C NMR)

As glucose is an alternate energy source for *S. vortens* (see chapter 5), long incubations with $2\text{-}^{13}\text{C}$ NMR glucose were required for the compound to be metabolised. Thus, in 89% of all

experiments, cells lost viability before glucose was metabolised, presumably due to mechanical damage resulting from stirring. As a result, a small number of replicates were achieved for each experiment. When washed, non-proliferating cells were incubated with glucose under N₂ for 12h, duplicate experiments show that the major products of 2-¹³C glucose metabolism recovered from the supernatant were lactate (63%), ethanol (29%) and alanine (8%) (Fig. 6.2.A). Under 2% O₂, 2-¹³C glucose was converted to lactate (53%) and ethanol (47%) (Fig. 6.2.B) and under 5% O₂, to lactate (50%) and ethanol (50%) (Fig 6.2.C).

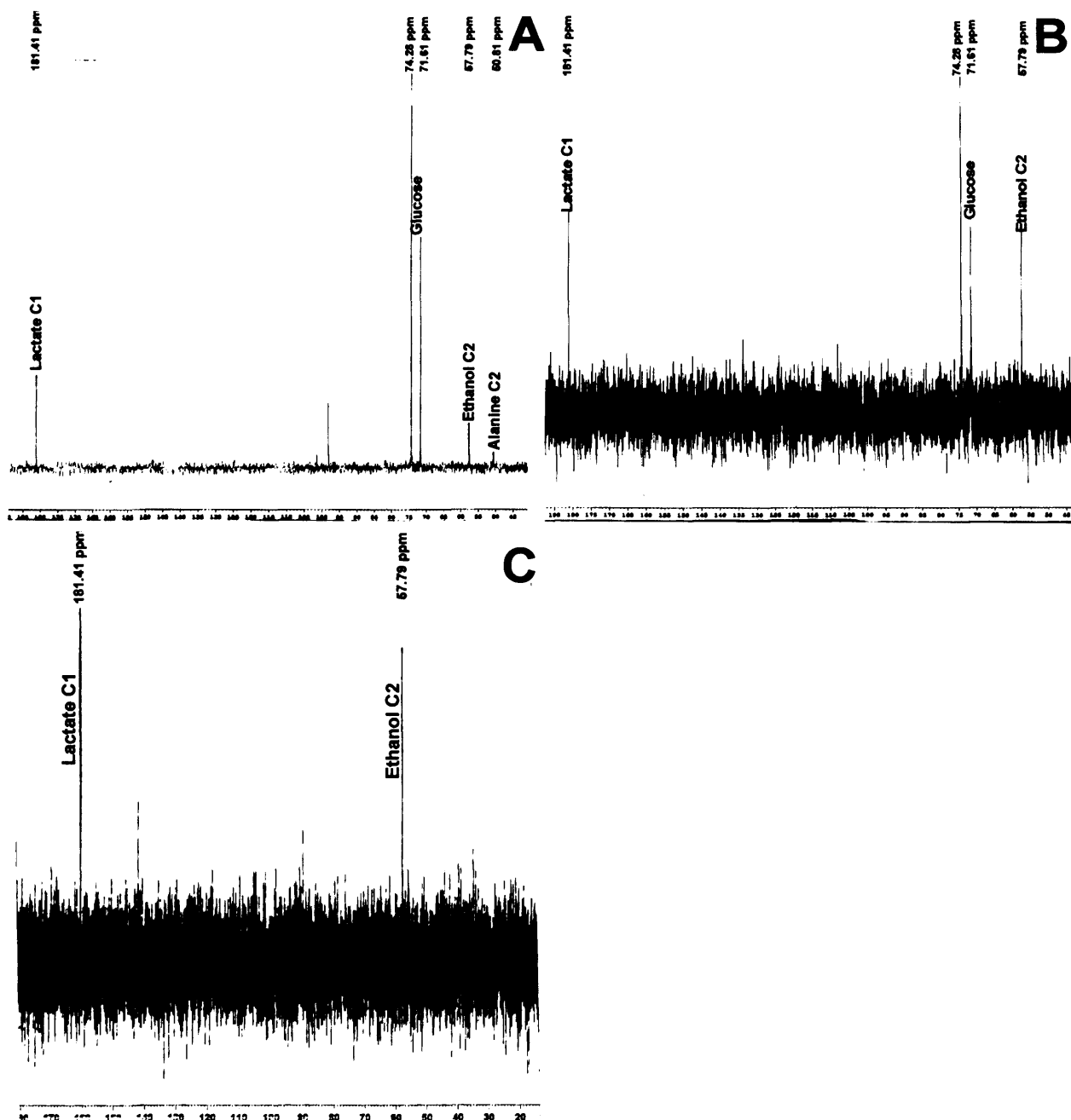


Fig 6. 2 Products of glucose metabolism in *S. vortens* at various O₂ tensions. A stirred suspension of log-phase trophozoites was incubated with 20mM 2- ^{13}C glucose, for 12h, under a constant flow of N₂ (A), 2%O₂ (B) or 5%O₂ (C). Spectra were recorded in H₂O at 62.9 MHz on a Bruker AV 250 MHz spectrometer, using $^2\text{H}_2\text{O}$ as an internal lock Figure 6. 2. A is the representative example of 2 experiments, figures 6. 2. A&B are single replicates.

6. 5 DISCUSSION

Results of the oxygen electrode experiment suggest that *Spironucleus vortens* possesses very efficient O₂ scavenging mechanisms and has a high tolerance (indicated by a high K_t), as well as a high affinity (indicated by low K_m) for the gas. However, values for K_m and K_t varied greatly between those experiments which used small or large steps in O₂ tensions. Indeed, both values for K_m and K_t appear much higher when large steps in O₂ tensions were used. While a degree of inaccuracy was expected in this experiment, due to the availability of few points for K_m determination (see Fig 6.1), such significant differences tend to indicate a flaw in experimental design. A low value of K_m indicates a high affinity for O₂; i. e., components of *S. vortens* can bind to O₂, even when it is present at very low concentrations. The much higher value for K_m, which was obtained when using large stepwise increases in O₂ concentrations (K_m=1.207 μM vs K_m=0.104 μM), suggests that detection of the low, minimum O₂ concentration at which *S. vortens* starts taking up O₂ was missed. The most accurate value for K_m is therefore likely to be the one obtained using small stepwise increases in O₂ concentrations (K_m= 0.104). The much higher value for K_t observed when using large stepwise increases in O₂ concentrations (K_t= 65.15 μM vs. K_t=6.78 μM), indicates that the threshold for oxygen inhibition is higher when large concentrations or oxygen are introduced quickly in the system, than it is when much smaller concentrations are added in small steps. This could be a sign of a physiological adaptation or oxygen-dependant metabolic switch, as can be observed in *Trichomonas foetus* (Lloyd, Williams et al. 1982). However, because these experiments require for cells to be stirred vigorously for over 4 h, mechanical damage to the cells, is as likely to explain the early cessation of O₂ consumption in the much longer, small step experiment. Besides, the ability of the organism to scavenge O₂ is probably determined by the availability of reduced cofactors, and as such, is undoubtedly limited in time. The duration of exposure to oxygen would therefore be an important factor in determining K_t. As in the “small step” experiments, it takes much longer to reach the same O₂ concentration than in the “large steps” experiments, obtention of much lower values for K_t in the former is not surprising., and the most accurate values for K_t would therefore be the ones obtained using large stepwise increases in O₂ concentration (K_t=65.15).

In comparison to that of other free-living or parasitic “anaerobic” protists, the Kt for O₂ in *S. vortens* is rather high (see Table 6. 3) (Biagini, Suller et al. 1997), indicating that this organism can tolerate exposure to high O₂ concentrations. Surprisingly, despite having such a high tolerance for oxygen, the organism has the ability to bind to the gas at very low concentrations, as proved by the extremely low value for Km. The combination of a very low value for Km and a high value for Kt, means that the organism can tolerate high concentrations of oxygen, but that some of its components are designed to bind to O₂ when present are very low concentrations. This is unusual, as in most protozoans, a high value for Kt is usually coupled to a high value for Km (Table 6. 3), both values being within the range of the oxygen concentrations encountered in the habitat (Biagini, Suller et al. 1997). The oxygen concentration in the hindgut of cichlids is unavailable in the literature, but is unlikely to be higher than that of the human intestine, and the high tolerance for oxygen therefore probably reflects the ability of the organism to colonise more aerobic environments, like the blood or the skin.

Table 6. 3. Apparent oxygen affinities (Km) and inhibition thresholds (Kt) of “anaerobic” protozoa from various habitats. (Adapted from Biagini *et al* 1997). ND= non determined.

Organism	Habitat	In situ O ₂ (μM)	Apparent Km for O ₂ (μM)	Kt for O ₂ (μM)
<i>Hexamita sp</i>	Free-living, limnic	0-30	12.97	100
<i>Metopus contortus</i>	Marine sediment	≤2.6	2.6	≤10
<i>Plagiopyla frontala</i>	Marine sediment	≤2.6	2.6	≤10
<i>Giardia lamblia</i>	Human small intestine	0-60	6.4	80
<i>Giardia intestinalis</i> *	Human small intestine	0-60	0.5-5.2	≥30
<i>Giardia muris</i>	Mouse small intestine	0-60	2.0	15
<i>Entamoeba histolytica</i>	Human large intestine	0-60	5.5	16
<i>Trichomonas vaginalis</i>	Human vagina	13-56	3.2	19
<i>Trichomonas foetus</i>	Bovine genitor-urinary tract	ND	1.6	ND
<i>Dasytricha ruminantium</i>	Rumen	≤0.25	0.33	ND

<i>Eudiplodinium magi</i>	Rumen	≤ 0.25	5.2	5
<i>Isotricha spp</i>	Rumen	≤ 0.25	2.33	ND
<i>Polyplastron multivesiculatum</i>	rumen	≤ 0.25	1.7	10
<i>Spironucleus vortens</i>	Hindgut of cichlids	ND	0.104	65.15

* Gives the range for seven strains of *Giardia intestinalis*

The very low value for K_m (0.104 μM) is puzzling. Indeed, although they are likely to encounter a similar range of O_2 concentrations, the value for K_m in *S. vortens* is much lower than that of other intestinal parasites of vertebrates (see Table 6. 3). Even more surprisingly, the K_m for O_2 in *S. vortens* is also significantly lower than that of rumen protists, which colonise environments where oxygen is lower than 0.25 μM , and were previously considered to possess the highest affinity for oxygen of all commensal protozoa (Lloyd, Williams et al. 1982; Ellis, Williams et al. 1989; Lloyd, Hillman et al. 1989). Previous mass spectrometric studies (see Chapter 4), have also shown that the rate of O_2 uptake ($62 \text{ nmoles} \cdot \text{min}^{-1} \cdot 10^7 \text{ cells}^{-1}$) in *S. vortens* was much higher than that of *T. vaginalis* or *H. inflata* ($4 \text{ nmoles} \cdot \text{min}^{-1} \cdot 10^7 \text{ cells}^{-1}$), which can themselves even rival that of organisms possessing mitochondrial cytochrome oxidases (Lloyd, Williams et al. 1982; Biagini, Suller et al. 1997; Biagini, Park et al. 2001).

There could be several physiological interpretations for such a high affinity for O_2 in *S. vortens*: First, it may reflect the need for O_2 detoxification, which would imply, that despite surviving exposure to high O_2 concentrations, the organism needs to remove even the smallest traces of the gas from its environment. This would in turn imply that *S. vortens* can tolerate high concentrations of O_2 for limited amounts of time, but that for optimal growth, strict anaerobiosis is needed. This is not very likely, as high density growth can be obtained in falcon tubes with a large headspace of air, and in which, despite the presence of reducing agents like cysteine, the culture medium would at least initially contain relatively high amounts of oxygen. Additionally, *S. vortens* has been shown to remain actively swimming after 12 h incubation in buffer at 25°C , when stirred under a constant flow of 5% oxygen (64 μM). In contrast, *G. intestinalis* was shown to lose its ability to scavenge oxygen and demonstrate loss of motility after 3-5 h at 25 μM O_2 (Lloyd, Harris et al. 2000), which

highlights the much higher tolerance of this organism to oxygen. Nevertheless, the hypothesis should be investigated by comparing the growth of cultures over several days in fermenters, at a range of O₂ tensions.

Alternatively, such a low K_m might not reflect the need for detoxification, but the utilization of molecular oxygen for metabolic processes. The intestinal tract of vertebrates is a complex and dynamic environment, where local concentrations of oxygen are likely to vary and may at time fall to very low levels (Pogrud and Steggerda 1948a&b). It is thus possible that in the event that some O₂ was required for metabolic processes, enzymes in *S. vortens* would have adapted to scavenging oxygen at very low concentrations. This hypothesis is particularly interesting, as it would infer that *S. vortens* may actually benefit from the presence of oxygen, and that, like *G. intestinalis* and *T. vaginalis*, it should be referred to as an microaerophile, rather than an aero-tolerant anaerobe. Again, in order to test that hypothesis, growth rates of the organism should be compared in a fermenter at a range of oxygen concentrations.

If K_m and K_t values for oxygen indicate that this organism can cope with important variations in O₂ concentrations, analysis of the end-products of glucose metabolism by ¹³C NMR reveals that such variations cause profound alterations in the carbon balance of this organism. Indeed, lactate, ethanol and alanine are the major products of glucose fermentation (Fig 6.2), however alanine is only produced under anaerobic conditions. Moreover, the ratio of lactate to ethanol decreases with increasing O₂ concentrations, going from 2.38 under anaerobic conditions to 1.13 at 5% O₂ and 1 at 2% O₂. The variation in fermentation end-products, according to oxygen concentration is a well described phenomenon and has also been reported in the intestinal parasite and closely related flagellate, *Giardia intestinalis* (Paget, Raynor et al. 1990; Lloyd and Paget 1991; Paget, Kelly et al. 1993). In that other parasite, ethanol, acetate and alanine are the main fermentations products, but alanine is also exclusively produced under complete anaerobiosis and increasing oxygen tensions cause a diminution in the ratio of ethanol to acetate production. In *Trichomonas vaginalis*, in which fermentations products are acetate, glycerol and lactate, increasing O₂ generates an increase in acetate accumulation (Paget and Lloyd 1990; Lloyd and Paget 1991). It has been speculated that such variations in the flux of carbon-containing metabolites may reflect changes in the

redox state of the cell. Indeed, proteins such as NADPH oxidase have been implicated in O₂ detoxification in “anaerobic” protists (Brown, Upcroft et al. 1995; Brown, Upcroft et al. 1996; Lloyd, Harris et al. 2000; Li and Wang 2006; Vicente, Testa et al. 2009). As availability of such redox components may be severely diminished in more aerobic environments, production of more oxidised metabolic end-products would become more likely. Similarly, in more anaerobic environments, renewal of NADP⁺ may become more costly in energetic terms, which would explain the production of more reduced end-products.

While the redox state of the cells is likely to influence the degree of oxidation of metabolic end-products, studies in *G. intestinalis* suggest that the picture is actually far from simple. Indeed, in this organism, acetate production has been shown not to originate from further oxidation of the ethanol produced from glucose fermentation, but to be derived from an entirely different pathway (Schofield and Edwards 1991; Paget, Kelly et al. 1993). This would imply that the same metabolic pathway is not just branched or extended, depending on the availability of redox cofactors, but that completely distinct pathways can be up-or down-regulated, according to O₂ concentrations. This may also be the case in *S. vortens*, as if no acetate was detected as a product of glucose metabolism in this study, ¹H NMR investigations of the products of glucose and endogenous metabolism show production of acetate in non-anaerobic suspensions (see Chapter 5). In order to identify the origin of the different metabolic products produced at various O₂ concentrations, further investigations using a combination of ¹H NMR and ¹³C NMR will be necessary. This will contribute in shedding some light on the mechanisms for the O₂-dependant tuning of the metabolism of this remarkably adaptable organism.

This study demonstrates that *S. vortens* can cope very efficiently with high O₂ tensions, but also possesses an unusually high affinity for the gas, which might indicate that it utilizes O₂ for metabolic processes. The organism is also shown to adapt its fermentative metabolism, depending on the availability of the gas, thus demonstrating the ability for fine metabolic tuning. Such high level of adaptability, combined with metabolic plasticity undoubtedly contributes to its success as a parasite.

6. 6 REFERENCES

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CHAPTER 7: EFFECT OF GARLIC AND ALLIUM-DERIVED PRODUCTS ON GROWTH AND METABOLISM OF *SPIRONUCLEUS VORTENS*

7.1 ABSTRACT

Spironucleus is a genus of small, flagellated parasites, which can infect a wide range of vertebrates and are a significant problem in aquaculture. As the use of metronidazole in food fish was banned due to toxicity problems, no satisfactory chemotherapies for the treatment of *Spironucleosis* are currently available. Using membrane inlet mass spectrometry and automated optical density monitoring of growth, we investigated the effect of *Allium sativum* (garlic), a herbal remedy known for its antimicrobial properties, on the growth and metabolism of *Spironucleus vortens*, a parasite of tropical fish and putative agent of hole-in-the-head disease. The allium-derived thiosulfinate compounds allicin and ajoene, as well as an ajoene-free mixture of thiosulfates and vinyl-dithiins were also tested. Whole, freeze-dried garlic and allium-derived compounds were found to have an inhibitory effect on gas metabolism, exponential growth rate and final growth yield of *S. vortens* in Keister's modified, TY-I-S33 culture medium. Of all the allium-derived compounds tested, the ajoene-free mixture of dithiins and thiosulfates was the most effective (MIC= 107 $\mu\text{g}.\text{ml}^{-1}$, IC_{50%}=58 $\mu\text{g}.\text{ml}^{-1}$), followed by ajoene (MIC= 83 $\mu\text{g}.\text{ml}^{-1}$, IC_{50%}= 56 $\mu\text{g}.\text{ml}^{-1}$); raw garlic (MIC>20 $\text{mg}.\text{ml}^{-1}$, IC_{50%}=7.9 $\text{mg}.\text{ml}^{-1}$) and allicin (MIC & IC_{50%}>160 $\mu\text{g}.\text{ml}^{-1}$) being significantly less potent. These concentrations are much higher than that reported to be required for the inhibition of most bacteria, protozoa and fungi investigated so far, indicating an unusual level of tolerance for allium-derived products in *S. vortens*.

7. 2 INTRODUCTION

Aquaculture is the fastest growing animal food production sector, and represented a \$63.3 billion global market in 2004 (Shehadeh and Maclean 1997). Stress and overcrowding of farmed fish render them particularly susceptible to infectious diseases, and pathogens are recognised to be the main cause of financial losses in aquaculture. For instance, in Asia alone, animal disease in aquaculture caused monetary losses of over \$3 billion in 1995 (Chua 1996).

The *Spirotrunculus* genus includes a range of parasitic anaerobic protozoan flagellates, and several species infect important species of farmed fish, having a crippling effect on their aquaculture. *S. barkhanus*, an opportunistic pathogen in salmonids, is indeed responsible for devastating outbreaks in salmon and trout farms in Norway and Canada (Mo, Poppe et al. 1990; Poppe, Mo et al. 1992; Poppe and Mo 1993; Sterud 1998; Sterud, Mo et al. 1998; Tojo and Santamarina 1998; Sterud, Poppe et al. 2003; Jorgensen and Sterud 2006), and infections may result in up to 100 % mortality (Guo and Woo 2004a&b). *S. vortens*, a warm-water species, is the suspected causative agent of hole-in-the-head disease in cichlids, a very popular family of ornamental, tropical fish (Nigrelli and Hafter 1947; O'Brien G, Ostland et al. 1993; Paull and Matthews 2001).

Although antimicrobial compounds have been employed heavily in aquaculture, chemotherapy of infectious diseases in this industry is generally difficult, as the effectiveness of antimicrobials tends to be limited in aquatic environments, especially in open, coastal systems (Shehadeh and Maclean 1997; Treves-Brown 1999). Metronidazole, a nitroheterocyclic antibiotic, is the drug of choice in the treatment of anaerobic infections. The compound itself has no antimicrobial activity, and must undergo reduction to be activated. As it has a low redox potential, it can only be activated by the lower redox-potential of the electron transfer systems present in anaerobic microbes. As a result, it is selectively toxic to anaerobic organisms (Edwards and Mathison 1970; Ings, McFadzean et al. 1974; Edwards 1980) and is used to combat infections caused by a range of anaerobic bacteria and protozoa, including *Trichomonas vaginalis* (Sikat, Heemstra et al. 1962; Streeter 1963; Grabowski 1976; Korner and Jensen 1976), *Giardia intestinalis* (Bassily, Farid et al. 1970; Majewska,

Kasprzak et al. 1991; Ikerd and Koletar 1993; Sousa and Poiars-Da-Silva 1999), as well as *Spironucleus* species (Tojo and Santamarina 1998; Sangmaneedet and Smith 1999). It is currently one of the most widely used drugs in the world, both in medicine and veterinary practice. However, it is genotoxic, mutagenic and carcinogenic in rats (Dobias, Cerna et al. 1994; Wesseling, Hahanau et al. 1996; Huet, Mortier et al. 2005; Maher, Youssef et al. 2008), and mutagenic and cytotoxic in fish (Cavas and Ergere Gozukara 2005), which prompted its ban in 1998 from use in aquaculture of European food fish by the Council Regulation 613/98/EEC (L82/14 1998).

Metronidazole is still employed in the aquarium trade, but its use is controversial in large scale aquaculture of ornamental fish, where anti-infectious drugs are often the last resort, due to their cost and the difficulty of administering the appropriate dose (Schelkle et al. 2009). Dispersal of this drug on a wide-scale in outdoor, ornamental fish farms, typically within food pellets, also raises serious environmental and health concerns. This drug is indeed quite water soluble, as well as non-biodegradable, and can therefore resist water and sewage treatments (Richardson and Bowron, 1985), which may result in long-lasting contamination of the aquatic environment. This is likely to have a devastating impact on the natural anaerobic microbial flora (Lanzky and Halling-Sorensen 1997) and may also encourage drug resistance (Chua 1996; Shehadeh and Maclean 1997). Metronidazole resistance has indeed been documented in *G. intestinalis* (Upcroft, Upcroft et al. 1990; Ellis, Wingfield et al. 1993; Borst and Ouellette 1995; Nash, Ohl et al. 2001; Upcroft and Upcroft 2001) and *T. vaginalis*, and the increased incidence of resistance to this important drug is a major public health issue (Upcroft, Upcroft et al. 1990; Rosenblatt 1992; Borst and Ouellette 1995; Freeman, Klutman et al. 1997; Land and Johnson 1999; Sobel, Nagappan et al. 1999; Upcroft and Upcroft 2001; Wendel 2003; Cohen and Desmond 2005). Wide scale contamination of aquatic environments with the drug is therefore potentially disastrous, as it may lead to generalised metronidazole resistance, not only in *Spironucleus* species, but also in other microbes (Diddle 1967; De Carneri, Achilli et al. 1969; de Carneri and Giannone 1971; de Carneri and Trane 1971; Meingassner, Havelec et al. 1978; Thurner and Meingassner 1978; Forsgren and Forssman 1979; Richardson and Bowron 1985; Krajden, Lossick et al. 1986; Tachezy, Kulda et al. 1993; Kulda 1999; Sobel, Nagappan et al. 1999). Such severe limitations in the use of

metronidazole highlight the need for alternative treatments of *Spirotrunculus* infections, in both food and ornamental fish.

Allium sativum, or garlic, a member of the liliaceae family, which originated from central Asia, is one of the edible plants that has been most referred to in medical folklore throughout history (Whitmore and Naidu 2000; Harris, Cottrell et al. 2001). For thousands of years, it has been considered by many cultures as a virtual panacea, and was attributed many properties, ranging from improving general health, to curing infections, boosting the immune system, protecting from the black plague and repelling evil spirits (Whitmore and Naidu 2000; Harris, Cottrell et al. 2001; Hunter, Caira et al. 2005). While at least one of these claims may be questionable, garlic has indeed been proven to improve cardiovascular health, reduce fat deposits and atherosclerosis, lower blood pressure and act as an antithrombic, as well as a potent antioxidant (Ernst 1987; Kleijnen, Knipschild et al. 1989; Neil and Silagy 1994; Agarwal 1996; Bordia, Verma et al. 1996; Yeh and Liu 2001; Sallam, Ishioroshi et al. 2004; Allison, Lowe et al. 2006; Budoff 2006; Morihara, Sumioka et al. 2006; Weiss, Ide et al. 2006; Benavides, Squadrito et al. 2007; Rahman 2007; Swiderski, Dabrowska et al. 2007). Perhaps one of the most interesting characteristics of garlic, however, is that it has powerful antimicrobial properties (Kabelik 1970; Ankri and Mirelman 1999; Harris, Cottrell et al. 2001; Hunter, Caira et al. 2005). As the world entered the era of antibiotic resistance, garlic generated an enormous amount of interest. It was shown to inhibit many pathogenic Gram-negative and Gram-positive bacteria, including some vancomycin-resistant enterococci and MRSA (Ankri and Mirelman 1999; Sasaki, Kita et al. 1999; Ross, O'Gara et al. 2001; Tsao, Hsu et al. 2003; Cutler and Wilson 2004; Iwalokun, Ogunledun et al. 2004; Gupta and Ravishankar 2005; Ruddock, Liao et al. 2005; Groppo, Ramacciato et al. 2007; Tsao, Liu et al. 2007). Garlic also demonstrated strong antifungal properties against pathogenic species, such as *Candida*, *Aspergillus* and *Cryptococcus* (Pai and Platt 1995; Shen, Davis et al. 1996; Yamada and Azuma 1997; Davis 2005; Fry, Okarter et al. 2005; Xia and Ng 2005), and proved extremely potent against many human viruses, even destroying HIV-infected cells *in vitro* (Tsai, Cole et al. 1985; Tatarintsev, Vrzheschch et al. 1992; Song, Song et al. 1997). It also exhibits antimicrobial activity against many parasitic protists, including *Trypanosoma*, *Leishmania*, *Leptomonas*, *Trichomonas* and *Entamoeba* (Mirelman, Monheit et al. 1987;

Reuter, Koch et al. 1996; Ankri, Miron et al. 1997), and was found effective in the treatment of *Giardiasis* (Soffar and Mokhtar 1991; Harris, Plummer et al. 2000; Harris, Cottrell et al. 2001; Harris, Plummer et al. 2001).

Many studies have attempted to breach the basis of the antimicrobial properties of garlic, however, the question is unexpectedly challenging, as the plant has a complex chemistry and contains hundreds of compounds, some of which are extremely unstable (Yoshida, Katsuzaki et al. 1999a&b; Avato, Tursil et al. 2000; O'Gara, Hill et al. 2000; Harris, Cottrell et al. 2001; Davis 2005; Ruddock, Liao et al. 2005; Xia and Ng 2005) and may act synergistically (Chung, Kwon et al. 2007). The majority of studies have initially focused on the many sulphur-containing compounds, whose unusual abundance bestows the plant its characteristic pungent smell. In the 1900s, analysis of steam extracts of crushed garlic showed that it contained several hundred of allyl-sulfide compounds. One of these, diallyl thiosulfinate, an oxygenated sulphur compound, also referred to as allicin, and first isolated by Cavallito and Bailey (1944), was long assumed to be the main antimicrobial in garlic (Whitmore and Naidu 2000; Huet, Mortier et al. 2005). Allicin is a very unstable, volatile compound with a pungent smell and is formed by the action of alliinase on a precursor molecule, alliin (Ankri and Mirelman 1999; Harris, Cottrell et al. 2001; Hunter, Caira et al. 2005). As alliin and alliinase are usually segregated, this process only occurs upon the crushing of garlic, and probably evolved as a localised defence mechanism against parasites and pathogens. The antimicrobial properties of allicin have been demonstrated. However, allicin-free garlic extracts retain activity against microbes (Fujisawa, Suma et al. 2008a) and several other compounds in garlic seem to also act as potent antimicrobials. Amongst these, the sulphonated compound vinyl-dithiin and ajoene, a product of the mild oxidation of allicin, have attracted the most interest, as they seem to generate a powerful, broad antimicrobial action, at concentrations sometimes lower than that of allicin (Tatarintsev, Vrzheschch et al. 1992; Bierer, Dener et al. 1995; Naganawa, Iwata et al. 1996; Yoshida, Iwata et al. 1998; Yoshida, Katsuzaki et al. 1999a; Whitmore and Naidu 2000).

Garlic, allicin and other allium-derived compounds have proven effective against several anaerobic protozoan flagellates, however their usefulness in the treatment of *S. vortens* has not been investigated and susceptibility of the organism to these compounds is

unknown. In this study, membrane inlet mass spectrometry and automated optical density monitoring was used to investigate the effect of whole garlic, allicin, ajoene and dithiin on the gas metabolism and growth of *S. vortens in vitro*, and compare it to that of metronidazole.

7.3 MATERIAL AND METHODS

Organisms and Cultures. *Spironucleus vortens*, ATCC 50386, were grown in TY-I-S33 medium as described in Chapter 2. Log phase cultures were harvested by centrifugation at 800 g for 2 min at room temperature in a bench centrifuge (MSE minor).

Inhibitors. Freeze-dried powder of whole Chinese garlic was obtained from Cultech, Ltd (Swansea, UK) and kept in a sealed bag at -30°C. HPLC analysis of this garlic by NEEM Biotech Ltd. revealed that the powder contained 0.223 % allicin, but no detectable amounts of ajoene. Stock solutions (20 mg. ml⁻¹) were prepared by dissolving the powder in PBS or culture medium, vortex mixing for 10 min and centrifuging at 3000g for 20 min. Solutions were then filtered through 0.2 µm porosity syringe filters, diluted as required in culture medium and used immediately. Metronidazole (Sigma) was dissolved in acidified buffer (final concentration 50 mM), slowly brought back to pH 6.8 with NaOH, filtered through a 0.2 µm porosity syringe filter, diluted as required and used immediately.

Allicin was produced by NEEM Biotech Ltd. according to the US patent 7179632 and HPLC analysis established its purity at 99%. Ajoene and dithiin were prepared by NEEM Biotech Ltd. according to the methods described by Block *et al.* (1986). Ajoene was produced by mild oxidation of a solution of pure allicin. Briefly, a solution of allicin was refluxed as a 10% solution in 3:2 acetone/water for 4 h, diluted with methanol and repeatedly extracted with pentane to remove non polar materials and then extracted with methylene chloride and concentrated. The resulting oily extract of ajoene was established to be 75% pure by HPLC. The remaining 25% consisted of allicin and various allicin breakdown products, which were not quantified. Dithiins were concentrated from raw garlic as described below. Chopped garlic pieces were soaked in methanol for 3 days. The concentrate was suspended in water and extracted with ether. The ether extract was concentrated, and the residue was stored at

25°C for 4 days as a 10% solution in methanol, suspended in water and repeatedly extracted with hexane to separate the less polar compounds from the more polar ones. The aqueous methanolic layer was then extracted with methylene chloride. Concentration of the hexane and methylene chloride extracts separately afforded yellow oils. The major non-polar components were thereby identified as diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide, allyl methyl trisulfide, 2-vinyl-4H-1,3-dithiin, 3-vinyl-4H-1,2-dithiin and allicin. Concentration of dithiins in the resulting oil was 32% by HPLC. Allicin, ajoene and dithiin were diluted in DMSO as required and used immediately.

Membrane inlet mass spectrometry. The effect of various concentrations of garlic, allicin and metronidazole on gas metabolism by *S. vortens* was investigated by membrane inlet mass spectrometry by measuring levels of dissolved O₂, CO₂ and H₂ in continuously stirred cell suspensions of log-phase trophozoites. Detailed methodology is given in Chapter 3. Log-phase trophozoites were harvested by centrifugation as described above, washed thoroughly in PBS (x3), resuspended in 5 ml PBS with 10 mM added glucose, and introduced into the reaction vessel. Inhibitors were added into the reaction vessel at the start of each experiment using a Hamilton syringe. In order to determine whether an individual inhibitor had an effect on gas production, one-way analysis of variance (ANOVA) and Fisher *a priori* analyses were performed on pooled data using Minitab 13 at a 95% confidence level.

Growth in Bioscreen C. Bioscreen C (LabSystems, Finland), an automated optical density-monitoring system, was used to monitor growth of *S. vortens* in 100 well honeycomb plates, in the presence of garlic, garlic derived compounds and metronidazole. Inhibitors were diluted in buffer, culture medium or DMSO (see above), added to 340 µl of Keister's modified TY-I-S33 culture medium, and 10 µl of homogenised log-phased cultures and introduced into triplicate wells. In order to avoid optical density differences between wells in the bioscreen experiments, garlic solutions were dissolved in culture medium and all dilutions of allicin, ajoene, dithiin and metronidazole were prepared so that the same volume of buffer or DMSO (0.35 µl) was introduced into all wells. For garlic and metronidazole experiments, triplicate control wells were filled with 340 µl of medium and 10 µl inoculate. As allicin, ajoene and

dithiin solutions contained DMSO, separate controls containing the same amount of DMSO were set up, and 3 control wells were filled with 339.7 μ l culture medium, 0.34 μ l DMSO and 10 μ l inoculate. Turbidity within wells was measured by OD on a vertical light path, using a wideband filter (420-580 nm) every 20 min for 120 h. Heat transfer fluid circulation maintained a constant temperature of 25°C and plates were shaken at low amplitude for 5 sec before each reading. OD measurements were logged, plotted against time, and exponential growth rates (μ), doubling times (T_d) and final yields were extrapolated from growth curves by applying the following formula:

$$\mu = 2.303(\log_{10}OD_{t_2} - \log_{10}OD_{t_1}) / (t_2 - t_1)$$

$$T_d = \ln 2 / \mu$$

$$\text{yield} = OD_{t_f} - OD_{t_0}$$

Original growth curves are included in Appendix 7. In order to determine whether medium dilution had an effect on exponential growth rates or final growth yield, one-way analysis of variance (ANOVA) and Fisher *a priori* analyses were performed on pooled data using Minitab 13 at a 95% confidence level. The lowest concentration of the compound to completely inhibit growth, or minimum inhibitory concentration (MIC) and the concentration at which the growth was reduced by 50% (inhibitory concentration at 50% or IC_{50%}) were derived from fitted curves of plots of total yield against inhibitor concentrations. Bioscreen outputs are presented in Appendix 7.

7. 4 RESULTS

7. 4. 1 Effect of freeze-dried garlic on metabolism and growth of *S. vortens*

Chinese garlic significantly inhibits O₂ consumption, CO₂ production and H₂ production of *Spironucleus vortens* at concentrations of 0.6 mg. ml⁻¹ and above (Table 7. 1). In the absence of an inhibitor, *S. vortens* exhibits a biphasic growth curve (see Chapter 2), and growth can therefore be characterised by the following parameters: exponential growth rate in the first log phase (μ_1), exponential growth rate in the second log phase (μ_2), and the total yield. Both growth rates are significantly reduced in the presence of garlic by 1 mg. ml⁻¹ and total yield is lowered by 10 mg. ml⁻¹ garlic. The lowest concentration of garlic to completely inhibit *S.*

vortens growth (MIC) was not determined in the current study, as it was above the highest concentration tested (20 mg. ml⁻¹ Fig. 7.3). An IC_{50%} of 7.9 mg. ml⁻¹ is derived from the fitted curve of total yields (Fig. 7.3).

Table 7. 1. Influence of freeze-dried powder of whole Chinese garlic on gas metabolism of *Spironucleus vortens*. Washed organisms were added to air-saturated PBS containing 10 mM glucose and 0.6 to 6 mg. ml⁻¹ garlic and introduced into a closed reaction vessel. The temperature was 20°C and the membrane interface of the probe was silicone rubber. The mass spectrometer was programmed to scan mass spectra repeatedly, collecting 128 spectra, before presenting data quasi-continuously at m/z values 2, 32 and 44, corresponding to peak values for H₂, O₂ and CO₂ respectively. Rates of O₂ consumption, CO₂ production and H₂ production were derived from data by linear regression analyses using Minitab 13. Significant differences from control rates are determined at a 95% confidence level using Fisher *a priori* analyses.

Garlic (µg. ml ⁻¹)	Allicin content (µg. ml ⁻¹)	Gas	Gaseous rate (nmoles.min ⁻¹ .10 ⁻⁷ cells)	SE	N	Inhibition	Significant difference? (ANOVA)
0	0	O ₂	74.06	6.39	4		
		CO ₂	75.30	11.21	3		
		H ₂	76.81	6.53	4		
600	1.34	O ₂	17.42	2.59	3	77%	Yes
		CO ₂	26.71	4.52	3	64%	Yes
		H ₂	13.56	6.78	3	82%	Yes
1200	2.68	O ₂	14.12	2.13	4	81%	Yes
		CO ₂	16.12	5.20	4	79%	Yes
		H ₂	7.28	4.42	4	91%	Yes
3200	7.14	O ₂	29.33	8.62	3	60%	Yes
		CO ₂	25.51	4.43	3	66%	Yes
		H ₂	13.38	6.62	3	83%	Yes
6000	13.38	O ₂	8.28	2.20	2	89%	Yes
		CO ₂	15.48	15.48	2	79%	Yes
		H ₂	0.82	0.49	2	99%	Yes

Table 7. 2. Influence of freeze-dried powder of whole Chinese garlic on growth of *Spironucleus vortens*. Exponential growth rates (μ_1 and μ_2) and total yield during the biphasic growth of *S. vortens* in Keister's modified TY-I-S33 culture medium were recorded by automated optical density monitoring (Bioscreen C) upon addition of 500 to 20000 $\mu\text{g. ml}^{-1}$ of garlic. Significant differences in exponential growth rates and total yields between various garlic concentrations and controls are determined at a 95% confidence level using a Fisher *a priori* analyses.

Garlic ($\mu\text{g. ml}^{-1}$)	Allicin content ($\mu\text{g. ml}^{-1}$)	Measurement		SE	N	Inhibition	Significant difference? (ANOVA)
0	0	μ_1 (h^{-1})	1.19E-01	1.41E-03	3		
		μ_2 (h^{-1})	3.90E-01	2.15E-02	3		
		Yield	1.06E+00	3.06E-03	3		
500	1.12	μ_1 (h^{-1})	3.90E-02	1.95E-02	3	67%	No
		μ_2 (h^{-1})	2.17E-01	8.13E-02	3	44%	No
		Yield	1.03E+00	1.29E-01	3	3%	No
1000	2.23	μ_1 (h^{-1})	4.60E-02	3.75E-02	3	62%	Yes
		μ_2 (h^{-1})	7.57E-02	5.28E-02	3	81%	Yes
		Yield	9.43E-01	2.44E-01	3	11%	No
5000	11.15	μ_1 (h^{-1})	0.00E+00	0.00E+00	3	100%	Yes
		μ_2 (h^{-1})	3.48E-02	1.17E-03	3	91%	Yes
		Yield	8.15E-01	9.54E-02	3	23%	No
10000	22.30	μ_1 (h^{-1})	0.00E+00	0.00E+00	3	100%	Yes
		μ_2 (h^{-1})	9.97E-03	5.09E-03	3	97%	Yes
		Yield	3.99E-01	2.17E-01	3	62%	Yes
20000	44.60	μ_1 (h^{-1})	0.00E+00	0.00E+00	3	100%	Yes
		μ_2 (h^{-1})	5.29E-03	3.12E-03	3	86%	Yes
		Yield	2.85E-01	1.56E-01	3	73%	Yes

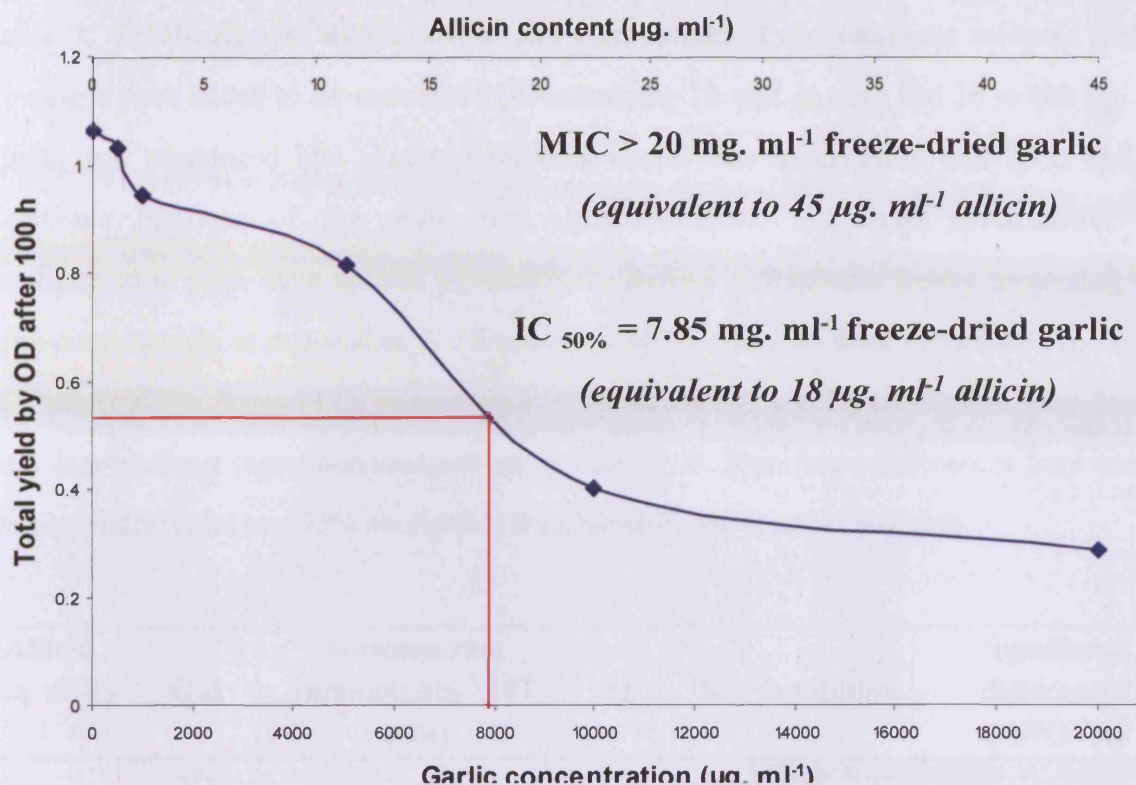


Fig 7. 3. MIC and IC 50% determination for freeze-dried garlic powder in *Spironucleus vortens*. Total yield by OD is plotted against garlic concentration (bottom axis), and a fitted curve is used to derive the lowest concentration to completely inhibit growth (MIC) and the concentration at which 50% of growth is inhibited (IC_{50%}). The allicin content in garlic powder is plotted on the top axis.

7. 4. 2 Effect of allicin on metabolism and growth of *S. vortens*

The effects of various concentrations of allicin on O₂ consumption, CO₂ production and H₂ production are presented in Table 7. 4 and their influence on growth are shown in Table 7. 5. Allicin concentrations of up to 50 µg. ml⁻¹ did not affect gas metabolism significantly. At 100 µg. ml⁻¹, allicin significantly reduces rates of O₂ consumption and H₂ production but CO₂ production is unaffected. Exponential growth rate is significantly decreased at concentrations of 10 µg. ml⁻¹ and above, and total yield is lowered from 40 µg. ml⁻¹. Figure 7. 6 shows that both MIC and IC_{50%} are above the highest concentration tested (160 µg. ml⁻¹).

Table 7. 4. Influence of allicin oil on gas metabolism of *Spironucleus vortens*. Washed organisms were added to air-saturated PBS containing 10 mM glucose and 10 to 100 $\mu\text{g. ml}^{-1}$ allicin, and introduced into a closed reaction vessel. The temperature was 20°C and the membrane interface of the probe was silicone rubber. The mass spectrometer was programmed to scan mass spectra repeatedly, collecting 128 spectra, before presenting data quasi-continuously at m/z values 2, 32 and 44, corresponding to peak values for H₂, O₂ and CO₂ respectively. Rates of O₂ consumption, CO₂ production and H₂ production were derived from data by linear regression analyses using Minitab 13. Significant differences from control rates are determined at a 95% confidence level using Fisher *a priori* analyses.

Allicin ($\mu\text{g. ml}^{-1}$)	Gas	Gaseous rate (nmoles.min ⁻¹ .10 ⁻⁷ cells)	SE	N	Inhibition	Significant difference? (ANOVA)
0	O ₂	31.47	9.34	6		
	CO ₂	106.89	15.28	5		
	H ₂	68.66	18.52	6		
10	O ₂	2.63	1.27	2	92%	No
	CO ₂	67.39	25.50	2	37%	No
	H ₂	2.87	2.74	2	96%	No
20	O ₂	26.12	10.65	2	17%	No
	CO ₂	91.02	90.64	2	15%	No
	H ₂	51.41	19.90	2	25%	No
50	O ₂	8.78	7.53	2	72%	No
	CO ₂	136.05	7.40	2	-27%	No
	H ₂	49.48	49.48	2	28%	No
100	O ₂	3.47	1.08	3	89%	Yes
	CO ₂	78.66	48.27	3	24%	No
	H ₂	4.82	4.54	3	93%	Yes

Table 7. 5. Influence of allicin on growth of *Spironucleus vortens*. Exponential growth rates (μ_1 and μ_2) and total yield during the biphasic growth of *S. vortens* in Keister's modified TY-I-S33 culture medium were recorded by automated optical density monitoring (Bioscreen C) upon addition of 5 to 160 $\mu\text{g. ml}^{-1}$ of allicin oil. Significant differences in exponential growth rates and total yields between various allicin concentrations and controls are determined at a 95% confidence level using Fisher *a priori* analyses.

Allicin Concentration (µg. ml ⁻¹)	Measurement		SE	N	Inhibition	Significant difference? (ANOVA)
0	µ 1 (h ⁻¹)	1.33E-01	1.68E-02	2		
	µ 2 (h ⁻¹)	3.29E-01	1.15E-01	2		
	Yield	1.02E+00	7.50E-03	2		
5	µ 1 (h ⁻¹)	9.86E-02	6.82E-03	3	26%	No
	µ 2 (h ⁻¹)	2.40E-01	2.74E-02	3	27%	No
	Yield	1.01E+00	1.01E-02	3	1%	No
10	µ 1 (h ⁻¹)	4.79E-02	2.96E-03	3	64%	Yes
	µ 2 (h ⁻¹)	1.63E-01	6.79E-03	3	51%	Yes
	Yield	9.82E-01	2.13E-03	3	4%	No
20	µ 1 (h ⁻¹)	4.85E-02	3.02E-03	3	63%	Yes
	µ 2 (h ⁻¹)	1.49E-01	3.18E-02	3	55%	Yes
	Yield	9.53E-01	4.00E-02	3	7%	No
40	µ 1 (h ⁻¹)	1.25E-02	1.52E-03	3	91%	Yes
	µ 2 (h ⁻¹)	3.42E-02	6.20E-03	3	90%	Yes
	Yield	8.19E-01	3.11E-02	3	20%	Yes
80	µ 1 (h ⁻¹)	1.34E-02	9.27E-04	3	90%	Yes
	µ 2 (h ⁻¹)	3.93E-02	2.74E-03	3	88%	Yes
	Yield	7.74E-01	6.91E-02	3	24%	Yes
160	µ 1 (h ⁻¹)	1.07E-02	1.08E-03	3	92%	Yes
	µ 2 (h ⁻¹)	3.49E-02	2.43E-03	3	89%	Yes
	Yield	8.39E-01	2.83E-02	3	18%	Yes

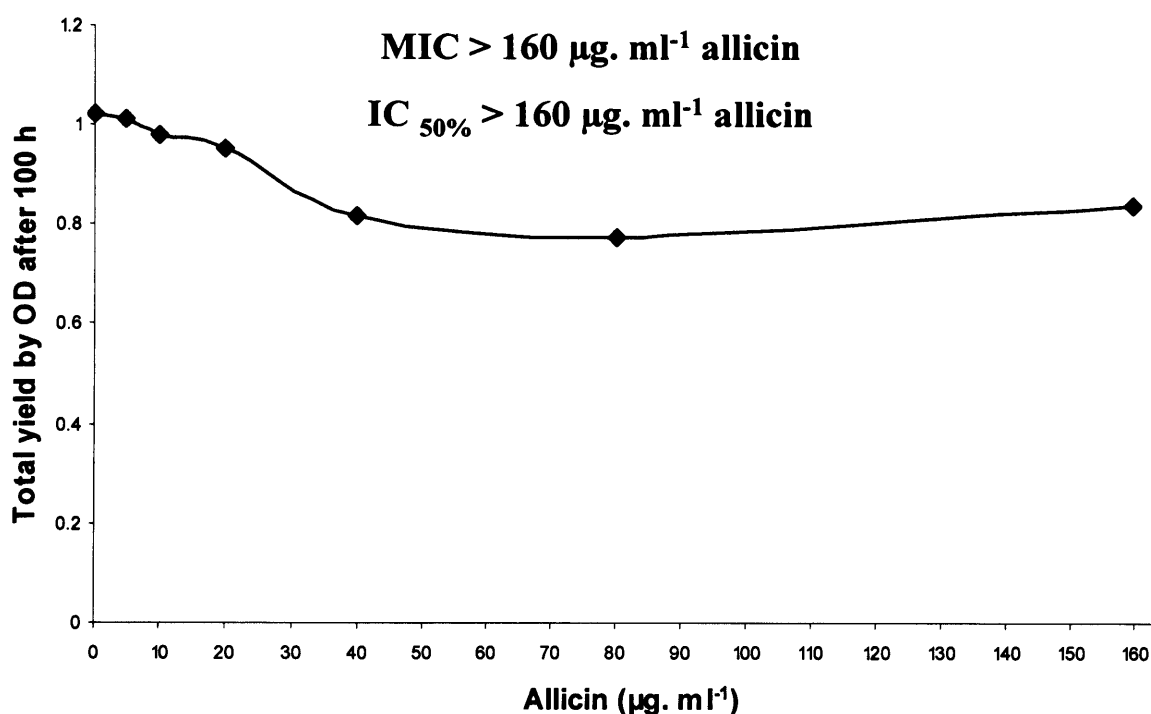


Fig 7. 6. MIC and IC 50% determination for allicin in *Spironucleus vortens*. Total yield by OD is plotted against allicin concentration, and a fitted curve is used to derive the lowest concentration to completely inhibit growth (MIC) and the concentration at which 50% of growth is inhibited (IC_{50%}).

7. 4. 3 Effect of crude ajoene extract on growth of *S.vortens*

Influence of crude ajoene oil on growth of *S. vortens* is presented in Table 7. 7. The oil significantly reduces the exponential growth rate on the first log phase from 5 µg. ml⁻¹, which corresponds to 3.75 µg. ml⁻¹ of pure ajoene. The exponential growth rate on the second log-phase is lowered at concentrations of 10 µg. ml⁻¹ of crude oil (equivalent to 7.50 µg. ml⁻¹ of pure ajoene) and above, and total yield is significantly reduced from 80 µg. ml⁻¹ (equivalent to 60 µg. ml⁻¹ of pure ajoene). An MIC of 107 µg. ml⁻¹ of crude ajoene extract is derived from the fitted curve of total yields (Fig. 7.8). This is equivalent to 80 µg. ml⁻¹ of pure ajoene. The IC_{50%} of crude ajoene extract derived from the fitted curve of Figure 7.8 is 58 µg. ml⁻¹. This is equivalent to 43.5 µg. ml⁻¹ of pure ajoene.

Table 7. 7. Influence of crude ajoene oil on growth of *Spironucleus vortens*. Exponential growth rates (μ_1 and μ_2) and total yield during the biphasic growth of *S. vortens* in Keister's modified TY-I-S33 culture medium were recorded by automated optical density monitoring (Bioscreen C) upon addition of 5 to 160 $\mu\text{g. ml}^{-1}$ of crude ajoene oil. Significant differences in exponential growth rates and total yields between various ajoene concentrations and controls are determined at a 95% confidence level using Fisher *a priori* analyses.

Crude Ajoene Oil Concentration ($\mu\text{g. ml}^{-1}$)	Ajoene Content ($\mu\text{g. ml}^{-1}$)	Measurement		SE	N	Inhibition	Significant difference? (ANOVA)
0	0	μ_1 (h^{-1})	1.33E-01	1.68E-02	2		
		μ_2 (h^{-1})	3.29E-01	1.15E-01	2		
		Yield	1.02E+00	7.50E-03	2		
5	3.75	μ_1 (h^{-1})	9.01E-02	7.19E-03	3	32%	Yes
		μ_2 (h^{-1})	2.52E-01	3.64E-02	3	23%	No
		Yield	1.05E+00	4.46E-03	3	-3%	No
10	7.5	μ_1 (h^{-1})	8.30E-02	9.17E-04	3	37%	Yes
		μ_2 (h^{-1})	1.77E-01	9.13E-03	3	46%	Yes
		Yield	1.00E+00	7.31E-03	3	2%	No
20	15	μ_1 (h^{-1})	9.00E-02	2.74E-03	3	32%	Yes
		μ_2 (h^{-1})	1.77E-01	4.27E-03	3	46%	Yes
		Yield	1.05E+00	6.07E-03	3	-3%	No
40	30	μ_1 (h^{-1})	8.04E-02	3.18E-03	3	39%	Yes
		μ_2 (h^{-1})	1.67E-01	1.72E-02	3	49%	Yes
		Yield	9.74E-01	8.72E-03	3	5%	No
80	60	μ_1 (h^{-1})	1.18E-02	6.32E-03	3	91%	Yes
		μ_2 (h^{-1})	2.58E-02	5.16E-03	3	92%	Yes
		Yield	1.20E-01	5.34E-02	3	88%	Yes
160	120	μ_1 (h^{-1})	0.00E+00	0.00E+00	3	100%	Yes
		μ_2 (h^{-1})	9.45E-03	2.83E-04	3	97%	Yes
		Yield	1.87E-02	1.20E-03	3	98%	Yes

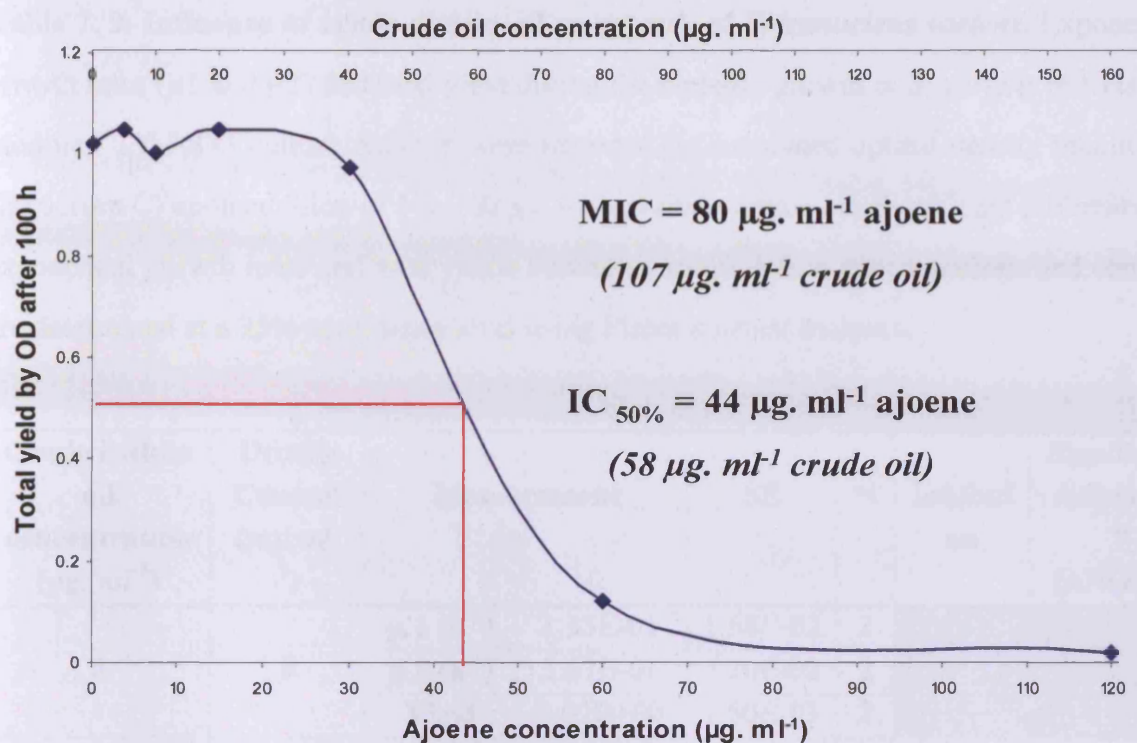


Fig 7. 8. MIC and IC 50% determination for crude ajoene extract in *Spironucleus vortens*. Total yield by OD is plotted against concentration of crude ajoene extract (top axis), and a fitted curve is used to derive the lowest concentration to completely inhibit growth (MIC) and the concentration at which 50% of growth is inhibited (IC_{50%}). Ajoene was produced from pure allicin by NEEM Biotech Ltd., as described by Block *et al.* (1986), and the crude oil employed contained 75% of pure compound. Pure ajoene concentrations contained in the crude extract are plotted on the bottom axis.

7. 4. 4 Effect of a mixture of dithiins and thiosulfinates on growth of *S. vortens*

Influence of crude dithiin oil on growth of *S. vortens* is presented in Table 7. 9. The oil significantly reduces both exponential growth rates from 5 µg. ml⁻¹ (equivalent to 1.60 µg. ml⁻¹ of pure dithiin), and lowers total yield at concentrations of 40 µg. ml⁻¹ and above (equivalent to 12. 8 µg. ml⁻¹ of pure dithiin). An MIC of 83 µg. ml⁻¹ of crude dithiin extract is derived from the fitted curve of total yields (Fig. 7.10). This is equivalent to 27 µg. ml⁻¹ of pure dithiin. The IC_{50%} of crude dithiin extract derived from the fitted curve of Figure 7.10 is 56 µg. ml⁻¹, which is equivalent to 18 µg. ml⁻¹ of pure dithiin.

Table 7. 9. Influence of crude dithiin oil on growth of *Spironucleus vortens*. Exponential growth rates (μ_1 and μ_2) and total yield during the biphasic growth of *S. vortens* in Keister's modified TY-I-S33 culture medium were recorded by automated optical density monitoring (Bioscreen C) upon addition of 5 to 160 $\mu\text{g. ml}^{-1}$ of crude dithiin oil. Significant differences in exponential growth rates and total yields between various dithiin concentrations and controls are determined at a 95% confidence level using Fisher *a priori* analyses.

Crude Dithiin oil concentration ($\mu\text{g. ml}^{-1}$)	Dithiin Content ($\mu\text{g. ml}^{-1}$)	Measurement		SE	N	Inhibiti on	Significant difference ? (ANOVA)
0	0	μ_1 (h^{-1})	1.33E-01	1.68E-02	2		
		μ_2 (h^{-1})	3.67E-01	7.70E-02	2		
		Yield	1.02E+00	7.50E-03	2		
5	1.60	μ_1 (h^{-1})	7.05E-02	2.11E-03	3	47%	Yes
		μ_2 (h^{-1})	1.82E-01	4.18E-02	3	50%	Yes
		Yield	1.03E+00	4.73E-03	3	-1%	No
10	3.20	μ_1 (h^{-1})	4.39E-02	2.67E-03	3	67%	Yes
		μ_2 (h^{-1})	1.22E-01	6.27E-04	3	67%	Yes
		Yield	1.01E+00	7.56E-03	3	1%	No
20	6.40	μ_1 (h^{-1})	4.45E-02	9.75E-03	3	66%	Yes
		μ_2 (h^{-1})	1.35E-01	7.50E-03	3	63%	Yes
		Yield	1.03E+00	7.41E-03	3	-1%	No
40	12.80	μ_1 (h^{-1})	2.99E-02	2.84E-03	3	78%	Yes
		μ_2 (h^{-1})	9.13E-02	2.02E-03	3	75%	Yes
		Yield	9.54E-01	7.71E-03	3	7%	Yes
80	25.60	μ_1 (h^{-1})	3.85E-03	3.85E-03	3	97%	Yes
		μ_2 (h^{-1})	2.76E-02	2.26E-03	3	93%	Yes
		Yield	3.80E-02	3.71E-03	3	96%	Yes
160	51.20	μ_1 (h^{-1})	0.00E+00	0.00E00	3	100%	Yes
		μ_2 (h^{-1})	5.96E-03	3.00E-03	3	98%	Yes
		Yield	1.30E-02	6.87E-03	3	99%	Yes

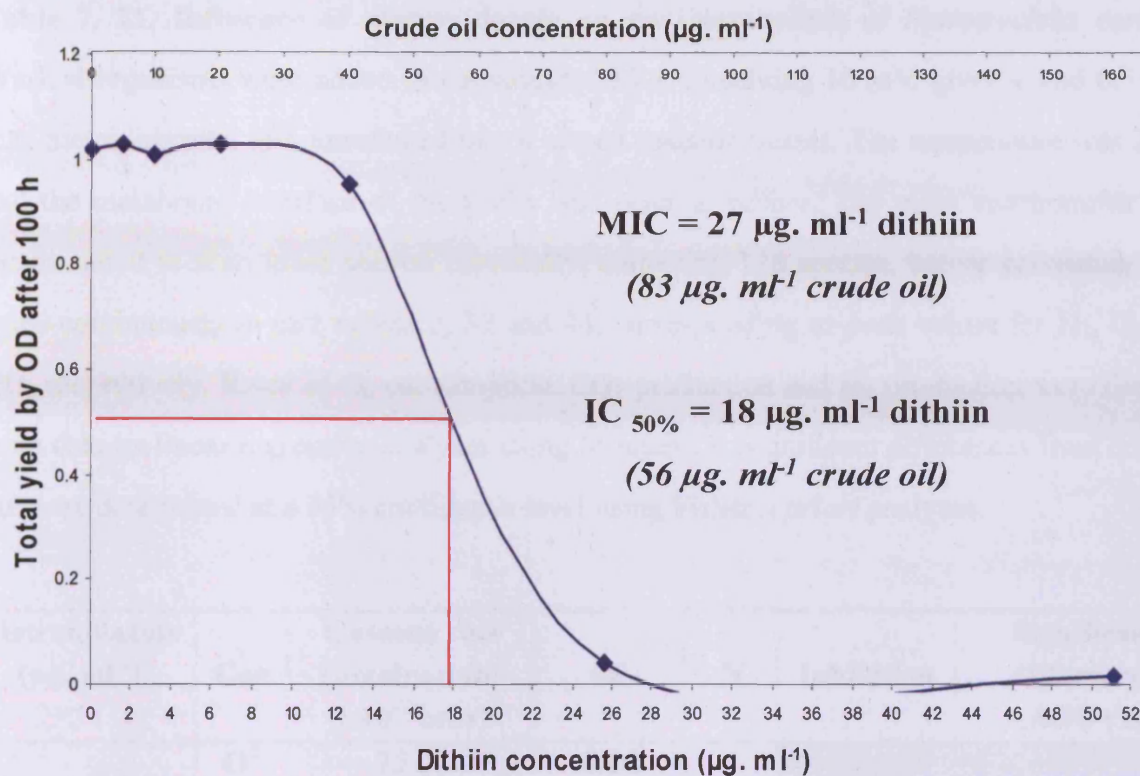


Fig 7. 10. MIC and IC 50% determination for crude dithiin extract in *Spironucleus vortens*. Total yield by OD is plotted against concentration of crude dithiin extract (top axis), and a fitted curve is used to derive the lowest concentration to completely inhibit growth (MIC) and the concentration at which 50% of growth is inhibited (IC_{50%}). Dithiins were concentrated from garlic by NEEM Biotech Ltd., as described by Block *et al.* (1986), and the crude oil employed contained 32% of pure compound. Pure dithiin concentrations contained in the crude extract are plotted on the bottom axis

7. 4. 5 Effect of metronidazole on metabolism and growth of *S. vortens*

The effects of metronidazole on gas metabolism and growth of *S. vortens* are presented in Tables 7. 11 and 7.12. Metronidazole inhibits H₂ production at concentrations of 85.6 µg. ml⁻¹ and above, and CO₂ production at concentrations of 171.2 µg. ml⁻¹ and above. O₂ consumption is not affected by up to 342.4 µg. ml⁻¹ metronidazole. Exponential growth rates and total yields of the organism are significantly reduced upon addition of 8.56 µg. ml⁻¹ metronidazole and above. Figure 7.13 shows that both MIC and IC_{50%} for metronidazole are under 8.56 µg. ml⁻¹, the lowest concentration tested.

Table 7. 11. Influence of metronidazole on gas metabolism of *Spironucleus vortens*.

Washed organisms were added to air-saturated PBS containing 10 mM glucose and 0.5 to 2 mM metronidazole, and introduced into a closed reaction vessel. The temperature was 20°C and the membrane interface of the probe was silicone rubber. The mass spectrometer was programmed to scan mass spectra repeatedly, collecting 128 spectra, before presenting data quasi-continuously at m/z values 2, 32 and 44, corresponding to peak values for H₂, O₂ and CO₂ respectively. Rates of O₂ consumption, CO₂ production and H₂ production were derived from data by linear regression analyses using Minitab 13. Significant differences from control rates are determined at a 95% confidence level using Fisher *a priori* analyses.

Metronidazole ($\mu\text{g. ml}^{-1}$)	Gas	Gaseous rate (nmoles.min- 1.10^{-7} cells)	SE	N	Inhibition	Significant difference? (ANOVA)
0	O ₂	75.00	6.84	4		
	CO ₂	75.30	11.21	3		
	H ₂	76.81	6.53	4		
85.6	O ₂	34.86	14.05	3	54%	No
	CO ₂	48.74	17.26	2	35%	No
	H ₂	36.95	17.40	3	52%	Yes
171.2	O ₂	49.51	17.72	4	34%	No
	CO ₂	25.82	9.91	3	66%	Yes
	H ₂	24.29	10.55	4	68%	Yes
342.4	O ₂	56.51	3.37	3	25%	No
	CO ₂	16.67	2.19	3	78%	Yes
	H ₂	14.67	1.33	3	81%	Yes

Table 7. 12. Influence of metronidazole on growth of *Spironucleus vortens*. Exponential growth rates (μ_1 and μ_2) and total yield during the biphasic growth of *S. vortens* in Keister's modified TY-I-S33 culture medium were recorded by automated optical density monitoring (Bioscreen C) upon addition of 0.05 mM to 2 mM metronidazole. Significant differences in exponential growth rates and total yields between various metronidazole concentrations and controls are determined at a 95% confidence level using Fisher *a priori* analyses.

Metronidazole Concentration ($\mu\text{g. ml}^{-1}$)	Measurement		SE	N	Inhibition	Significant difference? (ANOVA)
0	μ 1 (h^{-1})	1.19E-01	1.41E-03	3		
	μ 2 (h^{-1})	3.90E-01	2.15E-02	3		
	Yield	1.06E+00	3.06E-03	3		
8.56	μ 1 (h^{-1})	0.00E+00	0.00E+00	3	100%	Yes
	μ 2 (h^{-1})	2.08E-02	6.59E-03	3	95%	Yes
	Yield	3.76E-02	6.61E-03	3	96%	Yes
17.12	μ 1 (h^{-1})	0.00E+00	0.00E+00	3	100%	Yes
	μ 2 (h^{-1})	2.12E-02	3.52E-03	3	95%	Yes
	Yield	3.61E-02	4.87E-03	3	97%	Yes
34.24	μ 1 (h^{-1})	0.00E+00	*	1	100%	Yes
	μ 2 (h^{-1})	5.06E-02	*	1	87%	Yes
	Yield	2.03E-02	*	1	98%	Yes
85.60	μ 1 (h^{-1})	0.00E+00	0.00E+00	3	100%	Yes
	μ 2 (h^{-1})	1.97E-02	9.07E-03	3	95%	Yes
	Yield	2.12E-02	6.03E-03	3	98%	Yes
171.20	μ 1 (h^{-1})	0.00E+00	0.00E+00	3	100%	Yes
	μ 2 (h^{-1})	2.40E-02	4.06E-03	3	94%	Yes
	Yield	2.43E-02	4.22E-03	3	98%	Yes
342.40	μ 1 (h^{-1})	0.00E+00	*	1	100%	Yes
	μ 2 (h^{-1})	7.82E-03	*	1	98%	Yes
	Yield	1.40E-02	*	1	99%	Yes

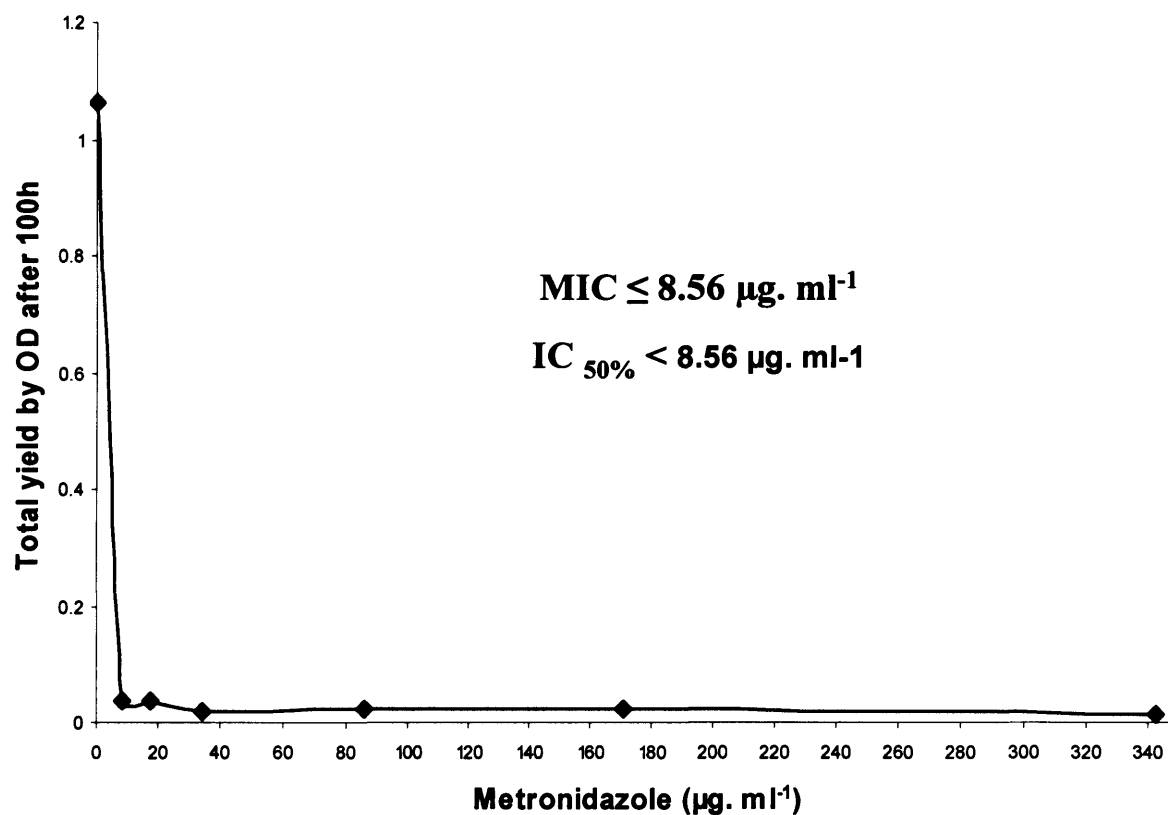


Fig 7. 13. MIC and IC 50% determination for metronidazole in *Spironucleus vortens*.

Total yield by OD is plotted against concentration of crude ajoene extract (top axis), and a fitted curve is used to derive the lowest concentration to completely inhibit growth (MIC) and the concentration at which 50% of growth is inhibited (IC_{50%}).

7.5 DISCUSSION

This study shows that garlic and allium-derived compounds have an inhibitory effect on *Spirotrunculus vortens*. Of all the allium-derived compounds tested, the ajoene-free mixture of dithiins and thiosulfinates was the most effective, followed by ajoene, raw garlic and allicin (see Table 7.13).

Table 7. 13. Comparison of the action of metronidazole, garlic and allium-derived compounds on *Spirotrunculus vortens*.

Inhibitor	MIC ($\mu\text{g. ml}^{-1}$)	IC_{50%} ($\mu\text{g.ml}^{-1}$)	Significantly reduces growth rate from ($\mu\text{g. ml}^{-1}$)	Significantly reduces final growth yield from ($\mu\text{g.ml}^{-1}$)
Freeze Dried Garlic	>20 000	7900	1000	10000
Allicin	>160	>160	10	40
Ajoene (75%) and allicin (25%)	107	58	10	80
Dithiins (32%) and ajoene-free mixture of thiosulfinates	83	56	5	40
Metronidazole	<8.56	<8.56	<8.56	<8.56

Garlic slows gas metabolism and growth of *S. vortens* at concentrations above 500 $\mu\text{g. ml}^{-1}$. However, extremely high concentrations (10 mg. ml^{-1} and above) are required to significantly affect the final growth yield after 80 h, suggesting that the lower concentrations of the compound only have a transient, static effect on *S. vortens*. IC_{50%} was 7.85 mg. ml^{-1} , but the MIC could not be determined, as the highest concentration tested (20 mg. ml^{-1}), did not completely inhibit growth after 80 h. This result is quite surprising, as much lower concentrations of garlic have been shown to completely inhibit the growth of the related protozoan parasite, *Giardia intestinalis* (MIC=0.30 mg. ml^{-1} , (Harris, Plummer et al. 2000; Harris, Plummer et al. 2001)) and administration of twice daily doses of 1 mg. ml^{-1} aqueous garlic for 72 h was shown to cure giardiasis and completely eliminate cysts from stools in

humans (Soffar and Mokhtar 1991). Variation in experimental design for MIC determination may have contributed to the stark difference in MIC values between *Spirotrunculus* and *Giardia*, as MIC values in *Giardia* were based on cell density after 24 h of growth, while in the current study, they were based on optical density of cultures after 80 h of growth (Harris, Plummer et al. 2000; Harris, Cottrell et al. 2001). As some of the many volatile compounds in garlic are known to be quite unstable in aqueous solutions (Freeman and Koder 1995; Fujisawa, Suma et al. 2008a&b), the length of growth incubation may indeed have had a strong influence on the values for MIC. Nonetheless, these results show that the use of a single dose of concentrated aqueous garlic would not be efficient in the treatment of *Spirotrunculosis*, and the effect of multiple doses over several subcultures should be investigated to assess potential usefulness of this plant in managing the parasite.

Allicin, believed to be the main antimicrobial in garlic (Cavallito and Bailey 1944), showed surprisingly little inhibitory effect on gas metabolism of *S. vortens*, and only inhibited O₂ consumption and H₂ production at the concentration of 100 µg. ml⁻¹. Even at this high concentration, CO₂ production was not significantly reduced. Growth rates were reduced from controls at concentrations equal or exceeding 10 µg. ml⁻¹. However, as with garlic, much higher concentrations (40 µg. ml⁻¹ and above) were required to significantly reduce the final yield, which suggests that at low concentrations, the action of the compound is also mainly transient and microbiostatic, rather than truly microbiocidal. MIC and IC_{50%} could not be determined, as the highest concentration of allicin tested (160 µg. ml⁻¹) did not completely inhibit growth or even reduce it by 50%. Again, this finding is rather surprising, as most bacteria investigated for sensitivity to allicin have IC_{50%} ranging from 3 to 15 µg. ml⁻¹ (Gupta and Ravishankar 2005), and antifungal activity is generally displayed between 0.3 and 32 µg. ml⁻¹ (Yamada and Azuma 1997; Ankri and Mirelman 1999; Whitmore and Naidu 2000; Davis 2005; Fry, Okarter et al. 2005). So far, only mucoid strains of some bacteria were found to have IC_{50%} above 100 µg. ml⁻¹ (Uchida, Takahashi et al. 1975; Ruddock, Liao et al. 2005), and such high level of resistance is probably due to the physical protection conferred by the mucus. *Spirotrunculus* appears to be unusually impermeable to fluorophores and chemical stains (Millet et al, unpublished data), possibly due to unusual properties of its membrane or pellicle. This may provide the protist with a degree of physical protection against allium-

derived compounds, thus explaining its unusually high level of tolerance towards the compound.

It should be noted that 7.85 mg of garlic (the $IC_{50\%}$ for this compound), contains 18 μg of pure allicin, however, the $IC_{50\%}$ of pure allicin was higher than 160 $\mu\text{g. ml}^{-1}$. Moreover, the concentration of allicin ranged from 0 to 100 $\mu\text{g. ml}^{-1}$ in the mass spectrometric analysis of gas metabolism, and from 0 to 160 $\mu\text{g. ml}^{-1}$ in the automated optical density monitoring of growth. These allicin concentrations are much higher than that contained in the whole garlic powder experiments (0 to 13.38 $\mu\text{g. ml}^{-1}$ for the mass spectrometry, and 0 to 44.60 $\mu\text{g. ml}^{-1}$ for the growth monitoring, see Figures 7.1 and 7.2), and yet they had considerably less inhibitory effect on gas metabolism, growth rates and final yields. This suggests that despite the long-standing belief that allicin is the main antimicrobial compound in garlic, the observed inhibitory properties of garlic on *S. vortens* are not due to allicin, or that the compound is only efficient synergistically.

Ajoene crude oil (75% pure, with 25% allicin and allicin breakdown products), was more efficient than allicin, as it reduced growth rates of *S. vortens* from 5 to 10 $\mu\text{g. ml}^{-1}$, which is equivalent to 3.75-7.5 $\mu\text{g. ml}^{-1}$ of pure ajoene oil (Table 7. 7). As for whole garlic and allicin, significant inhibition of growth yield after 80 h only occurred at much higher concentrations (80 $\mu\text{g. ml}^{-1}$ of crude oil, which corresponds to 60 $\mu\text{g. ml}^{-1}$ of pure ajoene). The MIC and $IC_{50\%}$ were respectively 107 $\mu\text{g. ml}^{-1}$ and 58 $\mu\text{g. ml}^{-1}$ of crude ajoene extract. This corresponds to 80 $\mu\text{g. ml}^{-1}$ and 44 $\mu\text{g. ml}^{-1}$ of pure ajoene. These values are much lower than that of either whole garlic or allicin (see Fig. 7. 8), and unless extraordinarily powerful synergistic effects are at play, the allicin content of the crude oil (25%), could hardly be deemed responsible for this stronger antimicrobial effect, as the much higher concentrations of pure allicin tested (Tables 7.4 and 7.5, Fig. 7.6) generated far less inhibition in *S. vortens*. However, if ajoene appears more efficient at inhibiting growth of *S. vortens* than either allicin or whole garlic, the values obtained for MIC of the compound are still considerably higher than that found in other parasitic protozoa and yeasts; 2.34 $\mu\text{g. ml}^{-1}$ for *Leishmania* (Ledezma, Jorquera et al. 2002) and 5.5 to 13 $\mu\text{g. ml}^{-1}$ for *Candida*, *Schizosacharomyces* and *Saccharomyces* (Yoshida, Kasuga et al. 1987; Naganawa, Iwata et al. 1996). Rather, they fall in range with the values obtained for the most resistant of Gram- positive bacteria (4 to 136

$\mu\text{g. ml}^{-1}$) and Gram-negative bacteria (116-152 $\mu\text{g. ml}^{-1}$) (Naganawa, Iwata et al. 1996; Ohta, Yamada et al. 1999; Whitmore and Naidu 2000).

The dithiin extract used in the current study was concentrated from chopped garlic, and its purity was only 32%. The remaining fraction was a mixture of diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide, allyl methyl trisulfide and allicin, but individual compounds were not quantified. As most of these compounds have been found to have some antimicrobial activity (Whitmore and Naidu 2000; Harris, Cottrell et al. 2001), it is difficult to assess the actual antimicrobial effect of dithiin here, but the mixture was slightly more efficient at inhibiting growth of *S. vortens* than the crude extract of ajoene. Growth rates of the organism were inhibited from 5 $\mu\text{g. ml}^{-1}$ of crude oil, and final yield was reduced significantly at a concentration of 40 $\mu\text{g. ml}^{-1}$ of the crude oil and above. MIC and IC_{50%} were 83 $\mu\text{g. ml}^{-1}$ and 56 $\mu\text{g. ml}^{-1}$ respectively, which is slightly lower than what is obtained for the crude ajoene extract. This small difference in antimicrobial activity may be attributed to the higher potency of dithiins, but may also be explained by the synergistic effect of the many antibacterial compounds present in the mixtures. As the antimicrobial activity of dithiins documented so far were lower than that of ajoene (MIC > 100 $\mu\text{g. ml}^{-1}$ vs. 15-20 $\mu\text{g. ml}^{-1}$ for ajoene against *Helicobacter pylori*, see Rie et al. 1999), the synergistic hypothesis seems more likely. However further work, using better defined and purer extracts, is required to address this question.

In comparison to allium-derived compounds, metronidazole completely inhibited growth rates and final growth yield at much lower concentrations (MIC and IC_{50%} being both well below 8.56 $\mu\text{g. ml}^{-1}$), which is far lower than the recommended peak serum concentrations for the treatment of Giardiasis in human (40 $\mu\text{g. ml}^{-1}$ when administered orally and 18-25 $\mu\text{g. ml}^{-1}$ by IV, (see Madanagopalan, Rao et al. 1975; Freeman, Klutman et al. 1997), and therefore confirms the susceptibility of the parasite to this drug (see Sangememeedet & Smith, 1999). Strangely, the much stronger inhibitory effect of metronidazole on growth rates and final growth yield of *S. vortens* is not reflected in its inhibition of H₂ and CO₂ production, which is in range with that of allicin. This may be due to the delayed action of the drug, which requires activation by the parasites, before exhibiting antimicrobial properties. The absence of inhibition of O₂ consumption during application of

metronidazole has already been described in *Trichomonas vaginalis* and (Gillin and Reiner 1982; Lloyd and Pedersen 1985; Yarlett, Yarlett et al. 1986; Ellis, Cole et al. 1992; Sousa and Poiars-Da-Silva 1999), and is likely due to the fact that the required activation of the drug can only occur under anaerobic conditions .

In conclusion, although this study established that all allium-derived compounds have an inhibitory effect on *S. vortens*, the potency of these compounds against this parasite was much lower than for most other protozoa, fungi and bacteria investigated so far. In sharp contrast to metronidazole, which had biocidal effects at very low concentrations, allium-derived compounds slowed the growth of *S. vortens* at low concentrations, but only inhibited total growth yield at much higher doses. This was particularly true of whole garlic, the concentration of which that was required to lower final growth yield being 10 times that needed to slow growth rates (versus 4 times for allicin and 8 times for ajoene and dithiins). Interestingly, MICs of ajoene and dithiins were less than twice as high as their IC 50%, while for whole garlic, the MIC value was significantly more than 2.5 times higher than the IC 50. Many aquariophiles believe that addition of 1-2% garlic in the feed enables to cure a variety of parasitic infections, however, to date, few conclusive studies have been conducted *in vivo*. In light of our investigations, it appears that such garlic concentrations would have little antimicrobial effect on *S. vortens* trophozoites within the intestine, especially as the acid conditions in the stomach are likely to destroy many of the active compounds in garlic (Freeman and Koder 1995; Fujisawa, Suma et al. 2008a&b). Higher doses of garlic, or purified thiosulfinates may prove more effective, however, an assessment of the toxicity of such doses for the fish is required to establish the potential usefulness of these compounds.

7. 6 REFERENCES

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CHAPTER 8: BOTH MITOSOME AND HYDROGENOSOME-LIKE ORGANELLES IN THE DIPLOMONAD PARASITE *SPIRONUCLEUS VORTENS*

8. 1 ABSTRACT

This study presents evidence of the presence of both mitochondria and hydrogenosome-like organelles in *Spironucleus vortens*, a parasitic diplomonad previously described as lacking redox-active organelles. Snap-freeze transmission electron microscopy of *S. vortens* trophozoites reveals the presence of small (100-150 nm), oval-shaped, organelles with a clear double membrane typical of mitochondria, and immunofluorescence microscopy shows that the mitochondrial proteins frataxin and Isu1 partially localise into small, discrete organelles, thus suggesting the presence of mitochondria-like organelles in *S. vortens*. Electron microscopy also demonstrates that the cytoplasm of *S. vortens* contains larger (200-500 nm), membranous structures with an electron-dense core, which resemble the hydrogenosomes of *T. vaginalis*. Moreover, confocal microscopy establishes that flavin-rich organelles of similar size, which possess an autofluorescence signature similar to that of mitochondria, are also present in *S. vortens*. Such organelles are also shown to concentrate rhodamine 123, TMRE and Mitotracker green. Finally, immunofluorescence-localisation of an Fe-Fe hydrogenase proved that the enzyme localises to large, discrete organelles, which, along with the EM, and other confocal data, provides strong evidence for the presence of a hydrogenosome-like, redox active organelle in *S. vortens*.

8. 2 INTRODUCTION

Spironucleus is a genus of small (typically 10 to 20 μM in length), eukaryotic parasites that belong to the suborder Diplomonadina, family Hexamita. Characteristically, they are pyriform-shaped, with two nuclei and a binary axial symmetry. Six flagella emerge at the front in groups of three, and two recurrent flagella run through the body and emerge posteriorly (Brugerolle, Joyon et al. 1973; Brugerolle, Kunstyr et al. 1980; Poynton and Morrison 1990; Poynton and Sterud 2002; Brugerolle, Silva-Neto et al. 2003). Perhaps the most defining structural characteristic of diplomonads is that, unlike most other eukaryotes, they are reported not to possess mitochondria or any other type of energy-generating organelle, such as hydrogenosomes.

The apparent lack of energy generating organelles and subcellular simplicity has long been considered an indication of the primitive origin of diplomonads, which were thought to have evolved prior to the endosymbiotic event (Cavalier-Smith 1987). This, as well as molecular studies based on ribosomal RNA genes (Sogin 1991; Sogin, Hinkle et al. 1993), initially led to the assignation of diplomonads to basal eukaryotic lineages along with the Microspora and Parabasalia. The basal position of these anaerobes within the eukaryotic tree is, however now increasingly questioned, due to identification of several genes of mitochondrial origin in numerous species of these taxa, including the Microspora, the parabasalia *Trichomonas*, and the diplomonads *Giardia* and *Spironucleus* spp. (Hashimoto, Sanchez et al. 1998; Roger, Svard et al. 1998; Roger, Morrison et al. 1999; Roger and Silberman 2002). The presence of these genes suggests that these organisms may have possessed a mitochondrion or mitochondrion-like structure, and lost it secondarily (Lloyd and Harris 2002). This hypothesis is further supported by the fact that no other molecular-based phylogenetic study has yet confirmed the primitive position of these three taxa as indicated by ribosomal RNA studies (Embley and Hirt 1998; Morin 2000). Several authors have thus advanced that the basal assignation of these organisms in ribosomal RNA trees might be an artefact caused by long-branch attraction, a phenomenon which is attributed to accelerated evolution (Morin 2000). This occurrence is well documented in molecular taxonomy; for instance, long-branch attraction in rRNA trees artificially leads to a closer position of

Drosophila melanogaster to nematodes rather than to other arthropods (Mason, Stage et al. 2009).

Discovery of a vestigial mitochondrion, or mitosome, in *Entamoeba histolytica* (Tovar, Fischer et al. 1999), another supposedly primitive, anaerobic parasite, further advanced the theory of a more recent origin for these “basal” eukaryotes. Mitosomes are small, double-membrane organelles, which contain typical mitochondrial proteins such as Hsp70, and biochemical characterisation of their enzymatic functions has identified them as vestigial mitochondria (Tovar, Fischer et al. 1999; Bakatselou, Beste et al. 2003; Regoes, Zourmpanou et al. 2005). These organelles are believed to have lost their energy-generating, oxidative phosphorylative functions during adaptation to the anaerobic lifestyle, but have retained their most essential function as a site for the maturation of Fe-S clusters (Kispal, Csere et al. 1999; Lill, Diekert et al. 1999; Lill and Kispal 2000; Muhlenhoff and Lill 2000). Following their discovery in 1999, and thanks to the availability of powerful, new TEM imaging techniques, such as snap-freeze cryosectioning, mitosomes were rapidly identified in several other “basal” eukaryotes, such as the microsporidians *Trachipleistophora hominis* (Williams, Hirt et al. 2002) and *Encephalitozoon cuniculi* (Vivares, Gouy et al. 2002), and the diplomonad *Giardia intestinalis* (Tovar, Leon-Avila et al. 2003). Currently, some form of mitochondrion-related organelle has been identified in all the protozoa and fungi that were originally described as amithochondriate (Embley and Martin 2006), the exception being *Spironucleus*; which has not yet been investigated using the more powerful imaging TEM techniques required to resolve these smaller organelles.

As new types of endosymbiotic organelles continue to be discovered and their function characterised, the distinction between hydrogenosomes, mitochondria and mitosomes has become blurred (Boxma, de Graaf et al. 2005; Martin 2005; Embley and Martin 2006; Stechmann, Hamblin et al. 2008) and much debate exists regarding the origin and evolution of all these double-membrane structures. The unifying function currently recognised in all forms of these double-membrane organelles appears to be the maturation of Fe-S clusters, and all mitochondria, hydrogenosomes and mitosomes described to date contain a partial or complete set of the Fe-S cluster assembly machinery proteins (Katinka, Duprat et al. 2001; Tachezy, Sanchez et al. 2001; LaGier, Tachezy et al. 2003; Tovar, Leon-Avila et al. 2003; Abrahamsen,

Templeton et al. 2004; Lill and Muhlenhoff 2005; van der Giezen, Leon-Avila et al. 2005; Goldberg, Molik et al. 2008; Lill and Muhlenhoff 2008; Lill 2009). Such proteins therefore appear good candidates for the immuno-investigations of potential mitosomes in the *Spironucleus* genus.

In this study, mitosome identification was attempted in *Spironucleus vortens*, a parasite of tropical fish, the ultrastructure of which has not yet been studied in detail. Immunolocalisation of frataxin, Isu1 and ferredoxin, mitochondrial and mitosomal proteins concerned with Fe-S cluster assembly (Lange, Kaut et al. 2000; Gerber, Muhlenhoff et al. 2003), was performed by fluorescence confocal scanning laser microscopy and snap-freeze TEM was used to investigate the presence of the small, double-membrane structures. As *S. vortens* produces large quantities of hydrogen (see Chapter 4), an atypical feat for an organism deprived of hydrogenosomes (Lloyd, Ralphs et al. 2002), immunolocalisation of an Fe-Fe hydrogenase was also undertaken.

8. 3 MATERIAL AND METHODS

Organisms and cultures. *Spironucleus vortens*, ATCC 50386, was obtained from Prof. J. Kulda (Charles University, Prague, Czech Republic) and cultured axenically at 20°C in Keister's modified TYI-S-33 medium as described in Chapter 2. Log phase cultures were harvested by centrifugation at 800 g for 3 min at room temperature in a bench centrifuge (MSE minor). *Giardia intestinalis*, JKH strain, a gift from Dr Timothy Paget (Medway, School of Pharmacy, UK), was isolated by Dr Victoria Hough. Trophozoites were cultured axenically at 37°C in Diamond's modified medium, as described in Chapter 3. After 10 min incubation of the tubes on ice, detached trophozoites were harvested by centrifugation (800 g, 5 min), at 4°C in a Beckman Coulter Avanti J-E centrifuge (Fullerton, California, USA). *Trichomonas vaginalis* (ATCC 30001) cultures were grown at 37°C in TYM medium. Log phase cultures were harvested by centrifugation at 800 g for 2 min at room temperature in a bench centrifuge (MSE minor).

Transmission electron microscopy. Log-phased trophozoites were harvested as described above, washed twice in PBS (pH 6.8), and fixed in 2% glutaraldehyde buffered in 0.05 mM PIPES (pH 7.2) containing 2 mM CaCl_2 and 0.2M sucrose for 4 h at room temperature. Cells were then rinsed in PIPES buffer, mixed into a 5% solution of warm (45°C) agar and left to set at 4°C. The agar was rinsed twice, post-fixed in 1% osmium tetroxide in PIPES at 4°C for 1h. Samples were then dehydrated in a series of ethanol solutions at 4°C (5 min in each 50%, 70%, x2 90%) followed by x4 immersions in 100% ethanol (5 min each) and x2 immersions in propylene oxide (5 min each) at 20°C. Infiltration was in araldite and ethanol, as follows: araldite-ethanol (1:1, v/v), 10 min, 20°C; araldite-ethanol (2:1, v/v), 10 min, 20°C; x2 araldite-ethanol (3:1, v/v), 10 min, 20°C; x3 pure araldite, 30 min, 20°C, pure araldite, 48 h, 60°C. Samples were then embedded in araldite containing 50% (w/w) dodycenyl succinic anhydride and 1.5 % (w/w) benzydimethylamine for 48 h at 60°C. The resin embedded blocks were cut into ultrathin sections (60nm thick) on a Reichert ultracut-E microtome (Reichert-Jung, Austria) using a diamond knife (Diatome, Agar Scientific Ltd., UK). The sections were mounted on pioloform-coated copper grids and counterstained in 2% uranyl acetate for 10 mins and Reynold's lead citrate (Reynold, 1963) for 5 mins prior to ultrastructural examination in a Philips EM 208 transmission electron microscope (FEI Co Ltd., Eindhoven, The Netherlands) at 80 kV.

Snap freeze transmission electron microscopy. Log-phased trophozoites of *S. vortens* were fixed in 0.5% glutaraldehyde or 4% paraformaldehyde/0.1% glutaraldehyde each in 0.2M PIPES buffer pH 7.2 for 30 min at room temperature, washed in PBS and stored at 4-8°C for 5 days. Cells were then pelleted (by Dr John Lucocq, University of Dundee), cryoprotected in 2.3 M sucrose in PBS and frozen on cryosectioning stubs in liquid nitrogen before being cryosectioned on a Leica ultracryomicrotome at 50-100 nm. Thawed cryosections were contrasted in methylcellulose/uranyl acetate and preparations were observed with a JEOL 1200EX transmission electron microscope at 80 kV.

Confocal microscopy. All preparations were viewed with a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope using a $\times 63$ or $\times 100$ objective. Z-stacks of optical sections taken

through entire organisms at a spacing of between 0.5 to 0.7 μm were used to generate three-dimensional maximum intensity projections. The LCS Lite software was used to edit pictures and perform quantitative analysis of fluorescence intensity. For the purpose of cellular context, some of the confocal projections were superimposed upon a transmitted light image obtained using Nomarski differential interference contrast optics. All buffer and solutions used for confocal microscopy were filtered through 0.2 μm porosity syringe filters, and cells were washed thoroughly in buffer (X3) prior to experiments. In order to reduce aldehyde-induced autofluorescence, all fixatives were prepared 24 h prior to experiment and kept at 5°C.

Lambda scans of autofluorescence signal. The fluorescence emission signal of discrete areas in non-stained, live, log-phased trophozoites, excited at 405, 453 and 488 nm, was recorded over 300 nm, in 10 nm increments.

Confocal microscopy of live cells incubated with cationic fluorophores. In order to detect potential redox organelles, cells were incubated with the commercially available fluorophores, rhodamine 123 (Invitrogen), Mitotracker green (Invitrogen) and tetramethylrhodamine (TMRE) (Invitrogen). Rhodamine 123 and TMRE are cationic fluorophores with low toxicity that accumulate on the negatively charged side of membranes and are thus commonly used to detect mitochondria or hydrogenosomes in live cells. The fluorescent probe, Mitotracker green, also accumulates into mitochondria, but also remains there even after loss of membrane potential, and can therefore be used for imaging of redox organelles in dead cells. Log phase cells were harvested as described above, incubated with each fluorophore and 10 mM glucose, washed 3 times in phosphate buffer before being embedded in low melting point agarose on a microscope slide to limit movement. Concentrations, loading times as well as excitations and emission wavelengths for all fluorophores are summarised in Table 8. 1.

Table 8. 1. Concentration, loading time and confocal laser settings used to view *Spironucleus vortens* trophozoites incubated with cationic fluorophores.

Fluorophore	Concentration	Loading time	Excitation wavelength (nm)	Emission wavelength (nm)
Rhodamine 123	500 nM	45 min	488	530
TMRE	100 nM	1.5 h	543	585
Mitotracker	100 nM	1.5 h	488	515
VF94	20 µg. ml ⁻¹	1.5 h	405	550

Immunoconfocal microscopy of cell suspensions. In order to detect potential redox-active organelles, antibodies raised against several hydrogenosomal, mitosomal and mitochondrial proteins from various species were applied to *S. vortens* trophozoites, and then conjugated to fluorescent secondary antibodies. The antibodies used are listed in Table 8. 2. Following the method of (Brugerolle, Bricheux et al. 2000), log-phase trophozoites were harvested by centrifugation as described above, washed in 5 ml PBS (pH 7.4) and fixed in phosphate buffer (pH 7.4) containing 3.5% (w/v) formaldehyde for 30 min at room temperature. The cells were then permeablized in phosphate buffer containing 0.5% Triton-X100 for 10 min, resuspended in 0.1 M glycine in phosphate buffer for 15 min and then in 1:20 goat serum at 4°C for 2 h. Cells were then incubated with 200 µl of the primary antibody (undiluted mAb supernatant) for 2 h at room temperature, and resuspended in 1 ml PBS for 30 min and harvested by centrifugation at 800 g for 3 min. This wash was repeated 3 times and cells were then incubated for 1 h with anti-mouse antibody conjugated with a fluorophore, as indicated in Table 8. 2. After final washes (×3) in 1 ml phosphate buffer, cells were mounted in Vectashield H-100 (Vector Inc., Burlingame). Selected samples were then counterstained for 1 min with 5µg. ml⁻¹ of FM 1-43 lipophilic styryl dye (Invitrogen). Negative control specimens were only treated with the secondary antibody. *Giardia intestinalis* or *Trichomonas vaginalis* were used as positive controls. In order to avoid potential ultrastructural damage caused by repeated centrifugations, immunofluorescence was also performed on gelatine-treated slides, as indicated in Table 8. 2. Slides were cleaned by immersion in 10% potassium hydroxide for 1 h, dipped in a solution containing 0.1 %

gelatine and 0.01 % chromium potassium sulphate at 70° C, and allowed to dry in a dust free environment. Following fixation in 3.5 % buffered (pH=7.4) formaldehyde, cells were mounted on slides and UV irradiated for 20 min to remove fixative-induced autofluorescence. Cells were then prepared for immunofluorescence as described in the above section, except that washes were performed by flooding the slide for 30 min in PBS.

Table 8. 2. Origin and conditions of utilisation of antibodies used in the current study

Antibody	Origin	Organism	Conjugated flurophore	Reaction in tube or on coated slide
α -subunit-succinyl-CoA synthetase	Hydrogenosome	<i>Trichomonas vaginalis</i>	FITC*	Tube
β -subunit-succinyl-CoA synthetase	Hydrogenosome	<i>Trichomonas vaginalis</i>	FITC*	Tube
Polyclonal anti-granule	Hydrogenosome	<i>Trichomonas vaginalis</i>	FITC*	Tube
Fe-hydrogenase	Hydrogenosome	<i>Blastocystis hominis</i>	FITC*	Tube
Frataxin	Mitosome	<i>Encephalitozoon cuniculi</i>	Alexa 488/Alexa 594	Coated slide, UV treated
Isu1	Mitochondrion	<i>Saccharomyces cerevisiae</i>	Alexa 488	Coated slide, UV treated
Ferredoxin	Mitosome	<i>Encephalitozoon cuniculi</i>	FITC*	Coated slide, UV treated

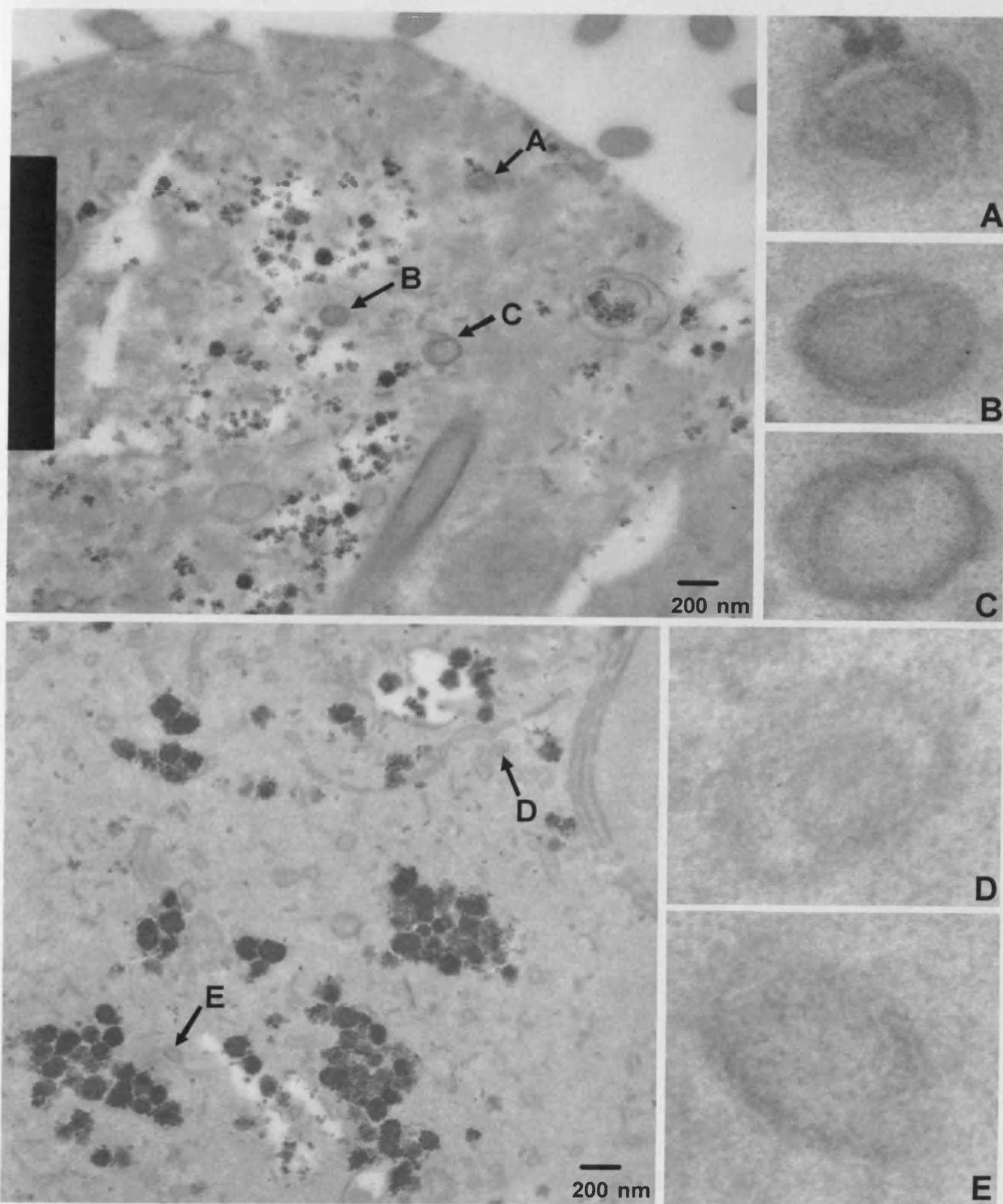
* fluoresceine isothiocyanate

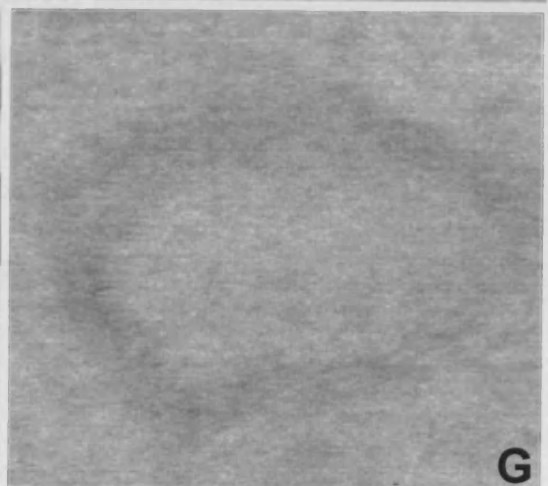
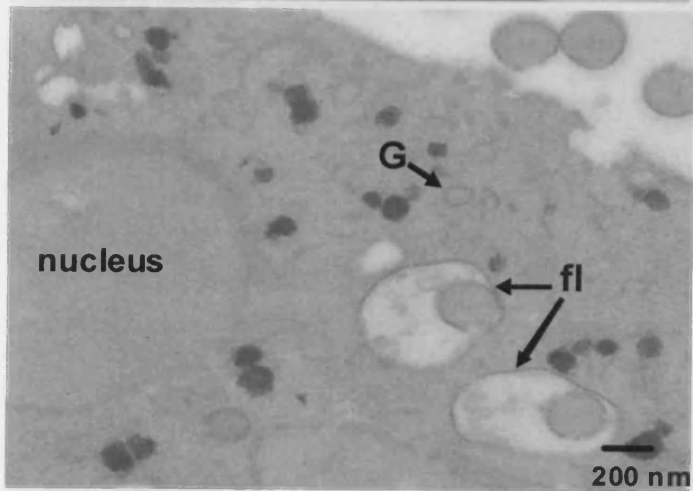
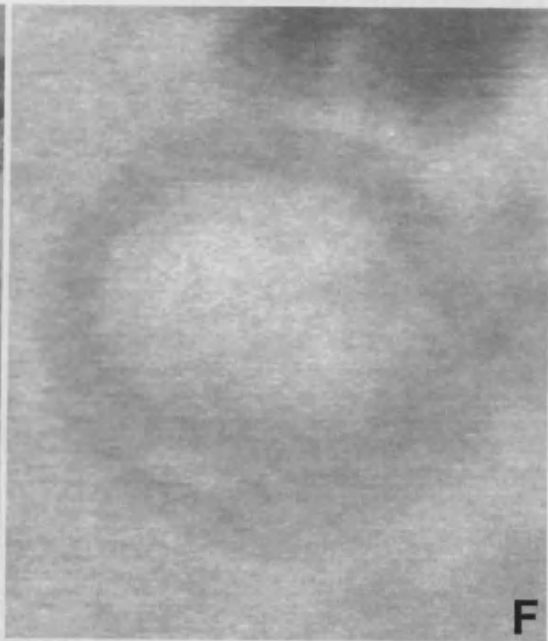
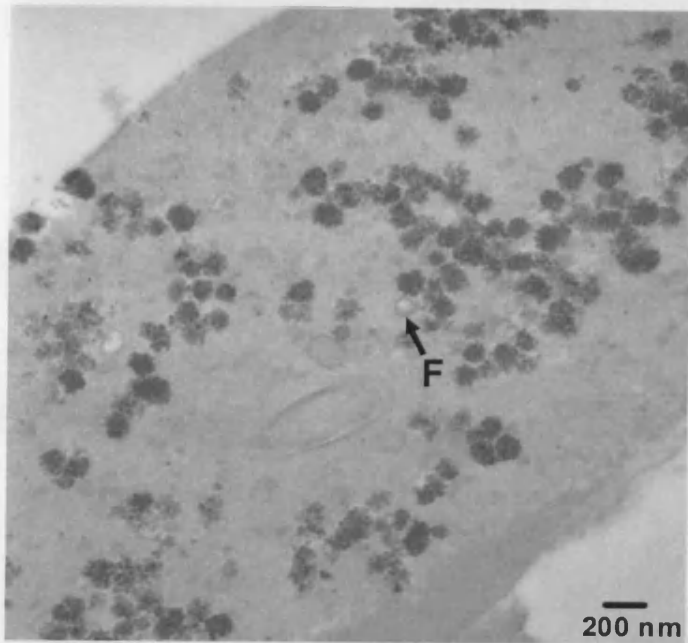
8. 4 RESULTS

8. 4. 1 Snap-freeze cryosectionning and standard TEM

Snap-freeze transmission electron microscopy reveals that *Spironucleus vortens* trophozoites contain small, oval shaped structures (100 to 150 nm in length) with a clear double membrane, typical of mitosomes (Figs. 8. 1A-G). Larger (200 to 500 nm), electron-dense,

structures (Figs. 8. 1H-J) resembling hydrogenosomes are also present. Resolution of the membrane of these latter structures is poor, but also suggests the presence of a double membrane (Fig. 8. 1I). The use of standard scanning electron microscopy with OsO₄ staining gives more contrast, but lower resolution of these cellular structures in both *T. vaginalis* (Fig. 8. 2) and *S. vortens* (Fig. 8. 3). In regular TEM, electron-dense hydrogenosomes of control, *T. vaginalis* sections appear clearly as electron-dense, spherical structures (Figs. 8. 2A-F). Although a double membrane can be detected in a few (Fig. 8. 2B), the resolution is too low in most cells. However, membrane folds are seen in some of the structures (Figs. 8. 2A-C). In *S. vortens*, putative hydrogenosomes appear as electron-dense spherical structures morphologically similar to hydrogenosomes of *T. vaginalis*. Double membranes can be resolved in these putative hydrogenosomes (Figs. 8. 3B, H and I), and as for *T. vaginalis*, membrane folds can be seen in some of the structures (see Fig. 8. 3C).





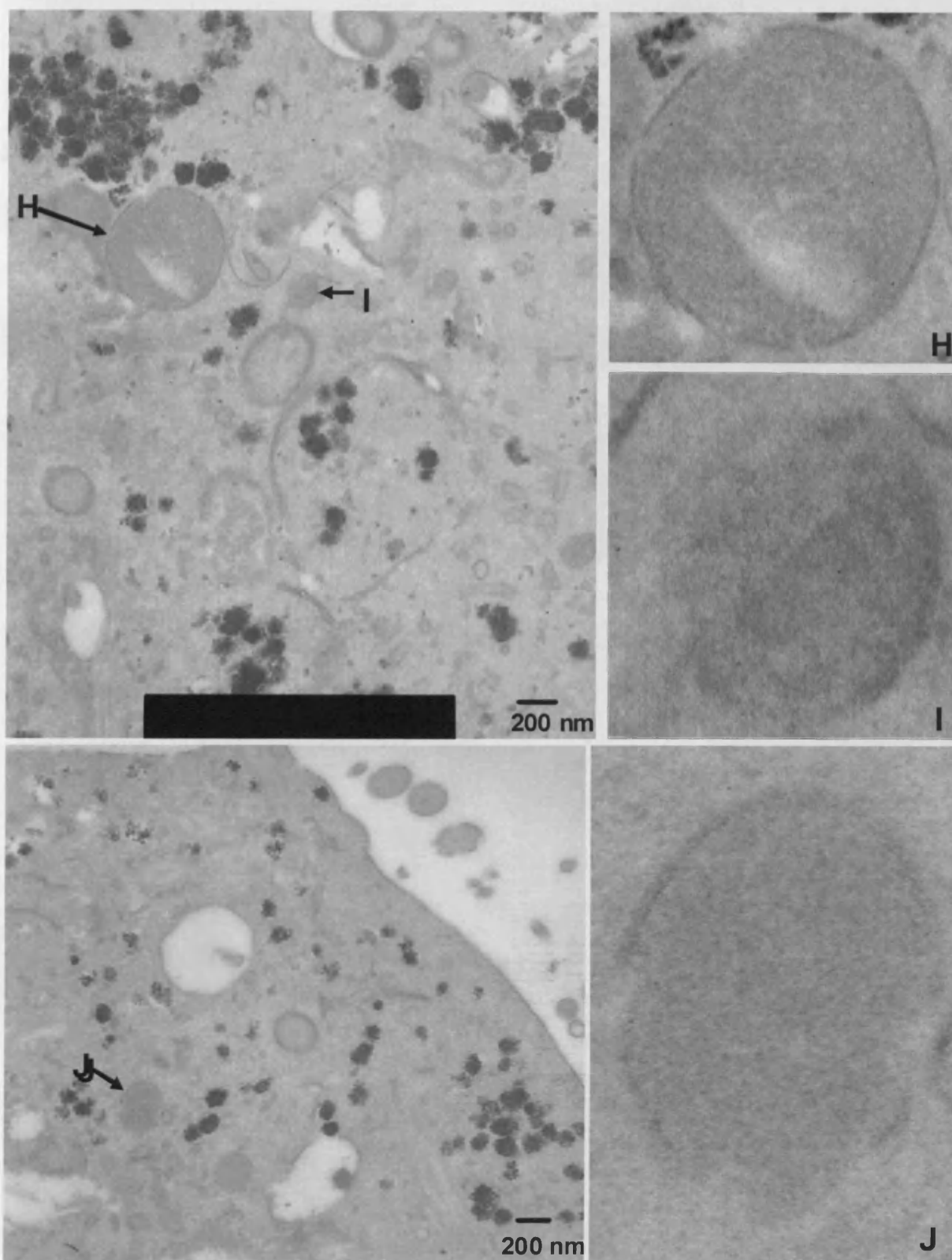


Fig. 8. 1. Snap-freeze transmission electron micrographs of *Spironucleus vortens* showing small (150 nm), oval-shape, double-membrane structures (A-F), as well as larger (200 to 500 nm), electron-dense, spherical structures (G-I). fl = flagellar sections. Digital magnifications of the area marked by arrows are showed on the right panel.

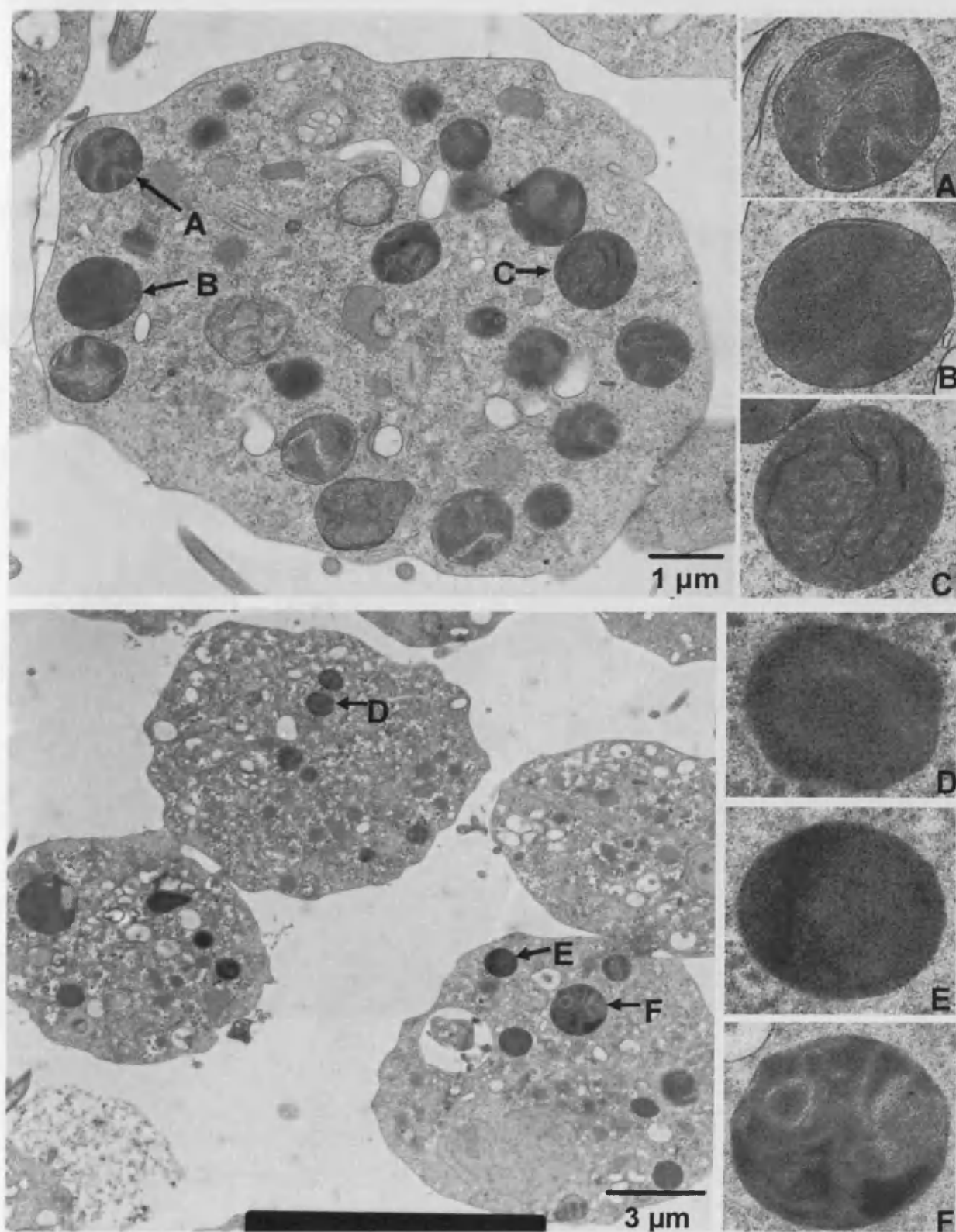
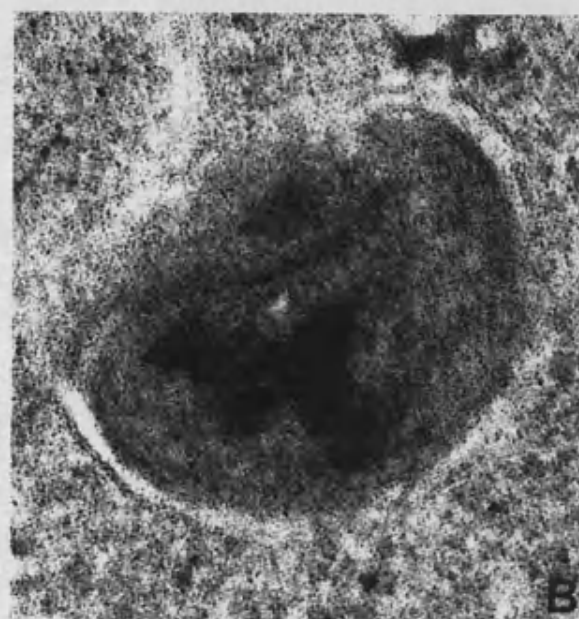
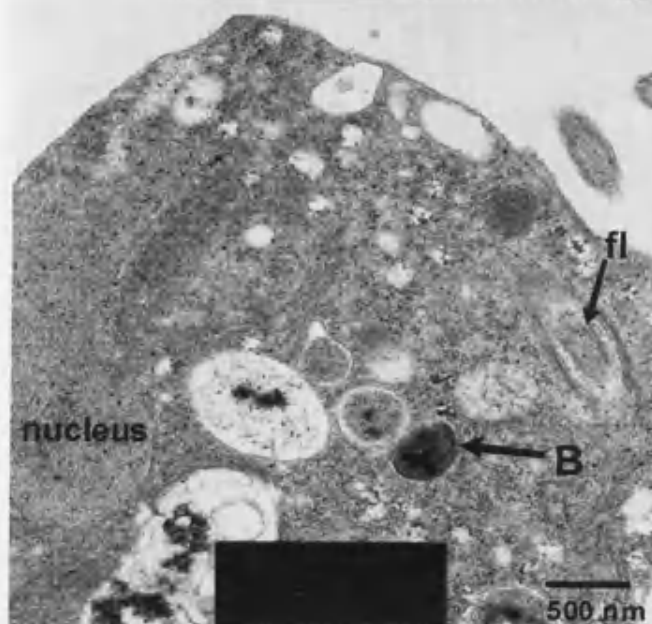
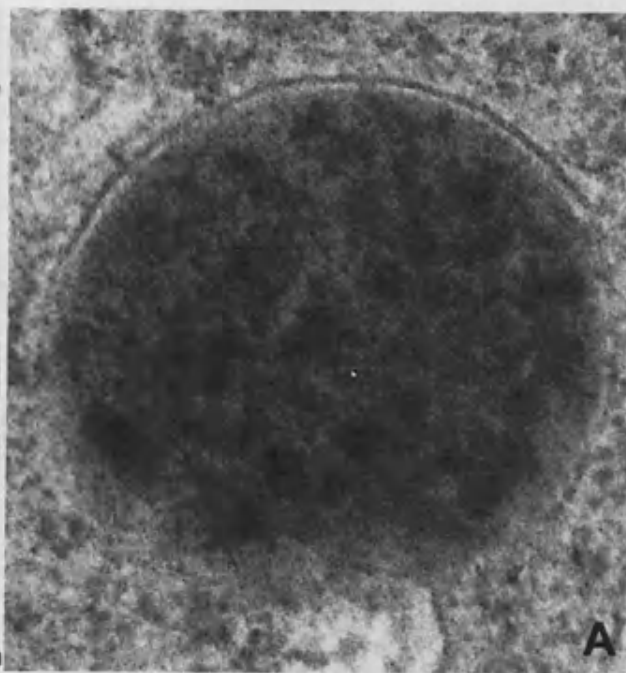
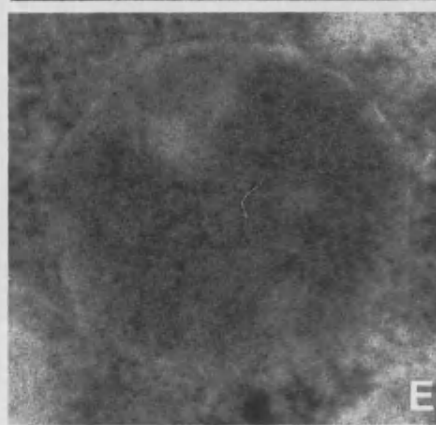
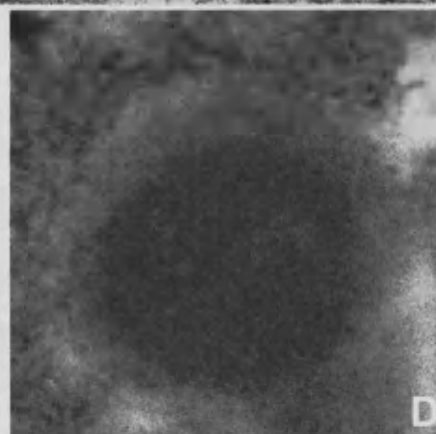
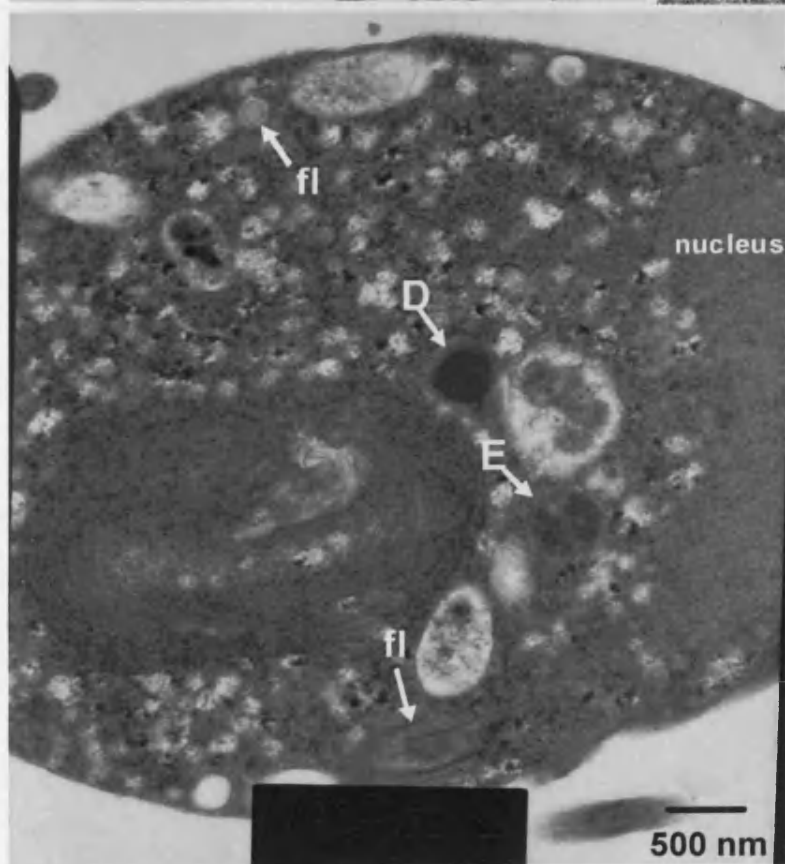
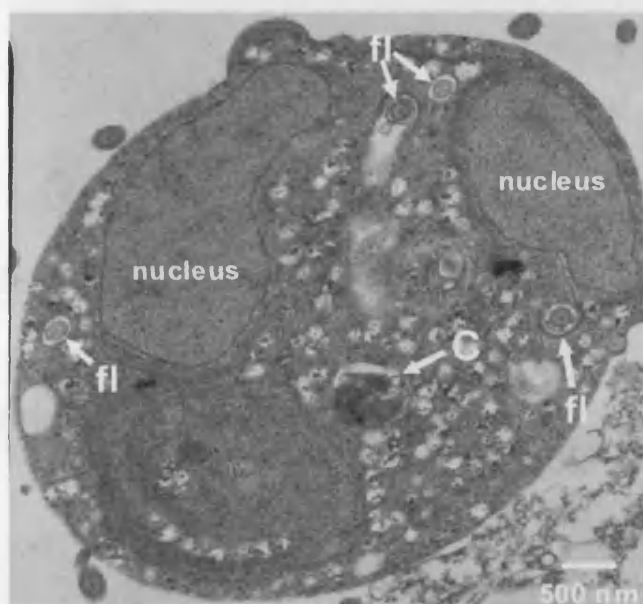


Fig. 8. 2. Transmission electron micrographs of *Trichomonas vaginalis*. Hydrogenosomes (A-F) appear as spherical, electron-dense structures of 0.9 to 1.6 μm in diameter. Digital magnifications of the area marked by arrows are showed on the right panel.





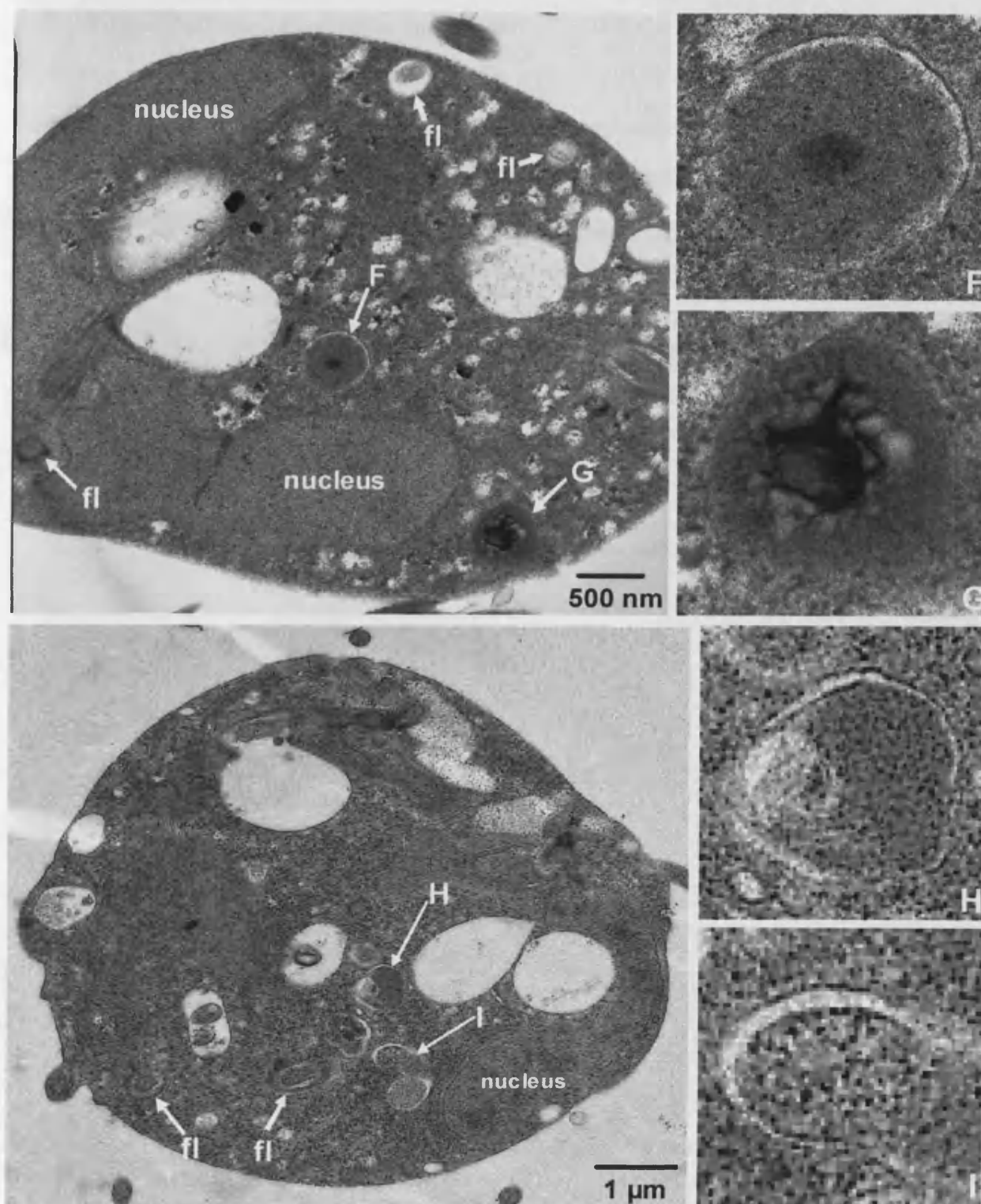
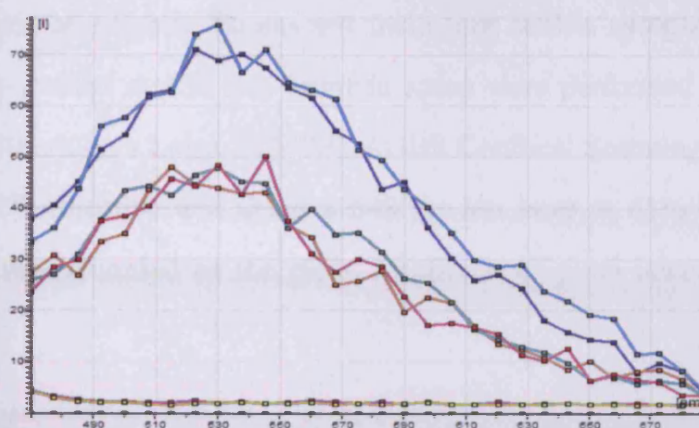
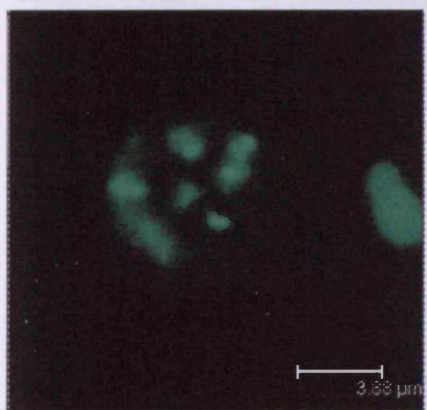
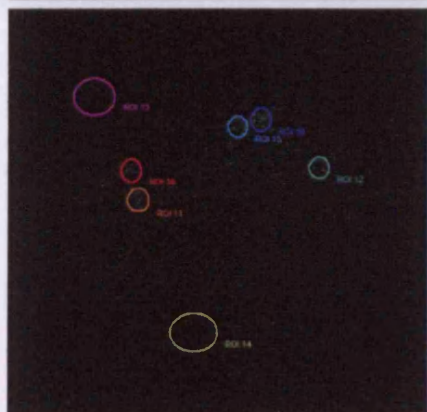
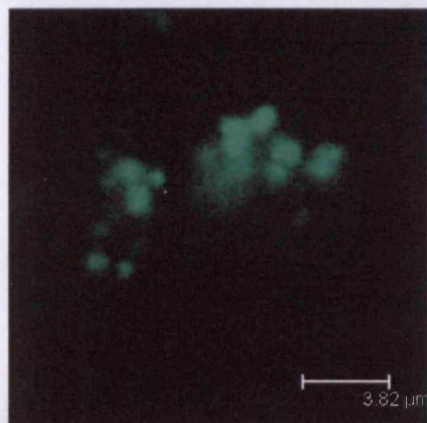


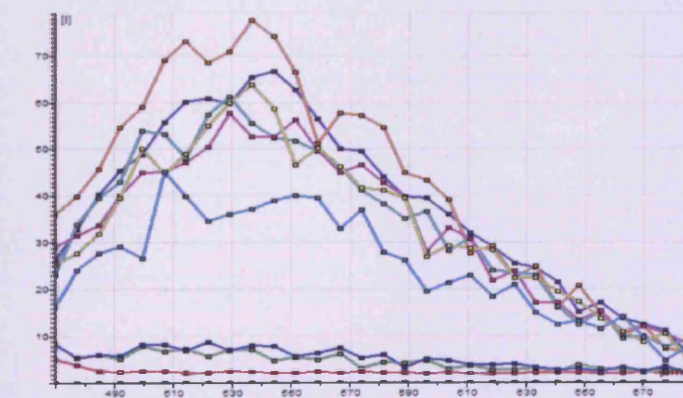
Fig. 8. 3. Transmission electron micrographs of *Spirotrunculus vortens* showing large (0.3 to 0.7 μm), electron-dense spherical structures (A-I). fl = flagellar sections. Digital magnifications of the area marked by arrows are showed on the right panel.

8. 4. 2 Analysis of autofluorescence spectrum

Excitation of unstained *S. vortens* trophozoites with 405, 458 or 488 nm laser line emission reveals numerous, brightly autofluorescent, spherical, cytoplasmic inclusions (Figs. 8. 4-7). Analysis of autofluorescence spectra of these particles shows that upon excitation at 458 nm, a strong emission signal is observed around 530 nm (Fig. 8. 4). When cells are excited with a 405 nm laser, fluorescence intensity is lower, and shifted slightly towards the blue (520 nm) (Fig. 8. 5), and when cells are excited at 543 nm, no significant fluorescent emission is observed (Fig. 8. 6). The use of a 488 nm excitation line also results in a strong signal, but significantly shifted towards the red (555 nm) (Fig. 8. 7).



ROI	ROI 10	ROI 11	ROI 12	ROI 13	ROI 14	ROI 15	ROI 16
#Pixel(ROI)	551.00	607.00	505.00	1830.00	2198.00	478.00	558.00
Area	788749.77 nm ²	846880.41 nm ²	704571.02 nm ²	2.55 μm ²	3.07 μm ²	664110.50 nm ²	778516.10 nm ²
Length	688.00 nm	688.00 nm	688.00 nm	688.00 nm	688.00 nm	688.00 nm	688.00 nm
Mean Amplitude	40.41	25.06	26.73	2.16	1.95	42.02	25.19
Max. Amplitude	72.38	48.96	48.77	4.53	4.31	77.31	51.10
Pos. Max. Ampl.	520.87 nm	513.60 nm	528.13 nm	470.00 nm	470.00 nm	528.13 nm	542.67 nm
Min. Amplitude	3.55	2.88	3.45	1.56	1.55	4.33	3.50
Pos. Min. Ampl.	680.73 nm	680.73 nm	680.73 nm	680.73 nm	658.93 nm	680.73 nm	680.73 nm
Average Deviation	19.16	11.92	13.20	0.35	0.29	18.73	12.92
Standard Deviation	22.18	14.06	15.29	0.57	0.53	21.76	14.99



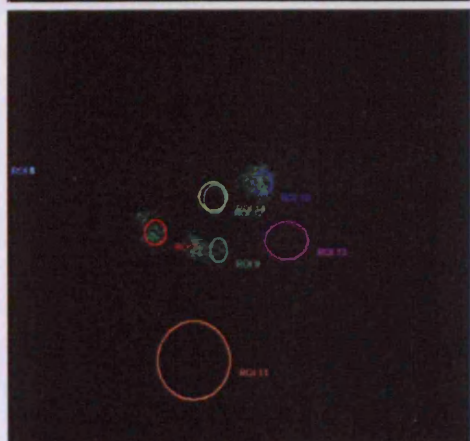
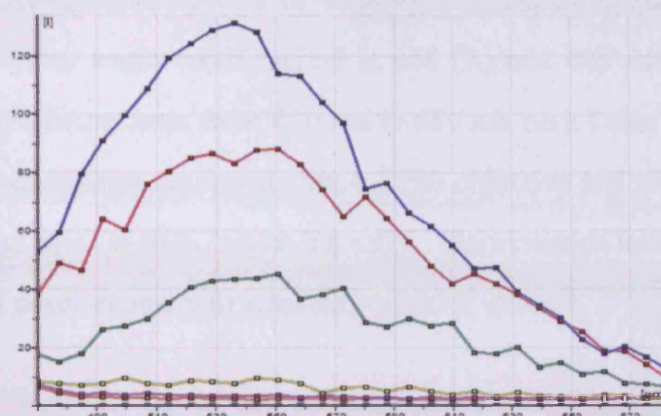
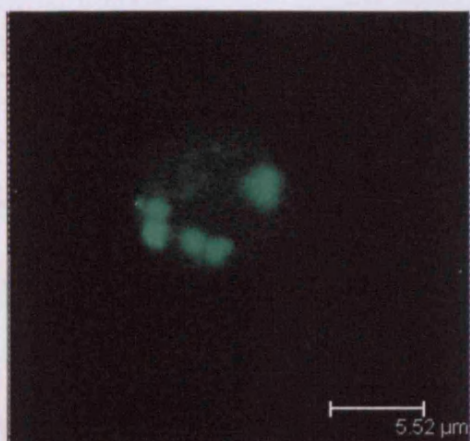
ROI	ROI 10	ROI 11	ROI 12	ROI 13	ROI 14	ROI 15	ROI 16	ROI 17	ROI 18
#Pixel(ROI)	484.00	315.00	407.00	292.00	345.00	291.00	3324.00	615.00	714.00
Area	695952.98 nm ²	452944.80 nm ²	585233.18 nm ²	419872.46 nm ²	496082.18 nm ²	418434.54 nm ²	4.78 μm ²	884320.41 nm ²	1.03 μm ²
Length	685.00 nm	685.00 nm	685.00 nm	685.00 nm	685.00 nm	685.00 nm	685.00 nm	685.00 nm	685.00 nm
Mean Amplitude	39.03	43.35	34.96	34.22	34.47	25.44	2.32	4.61	5.29
Max. Amplitude	67.95	79.16	62.36	58.86	65.10	46.12	5.07	8.19	8.76
Pos. Max. Ampl.	541.07 nm	534.50 nm	527.33 nm	527.33 nm	534.50 nm	505.83 nm	470.00 nm	470.00 nm	520.17 nm
Min. Amplitude	6.44	6.62	7.68	8.38	5.07	4.68	1.91	2.24	1.92
Pos. Min. Ampl.	677.83 nm	677.83 nm	670.67 nm	677.83 nm	677.83 nm	670.67 nm	613.33 nm	634.83 nm	677.83 nm
Average Deviation	15.83	19.57	14.57	13.70	14.46	9.80	0.34	1.59	1.82
Standard Deviation	18.61	22.90	17.04	15.91	16.89	11.56	0.63	1.86	2.13
Variance	346.30	524.62	290.36	253.23	285.22	133.60	0.40	3.47	4.55

A

B

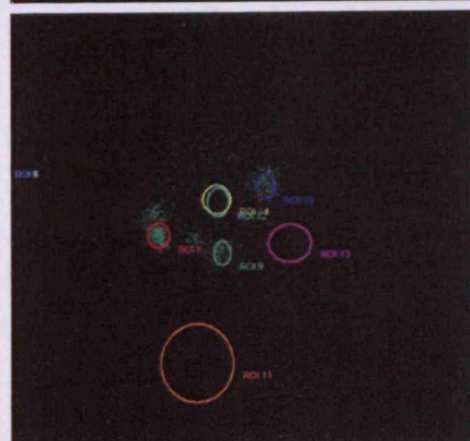
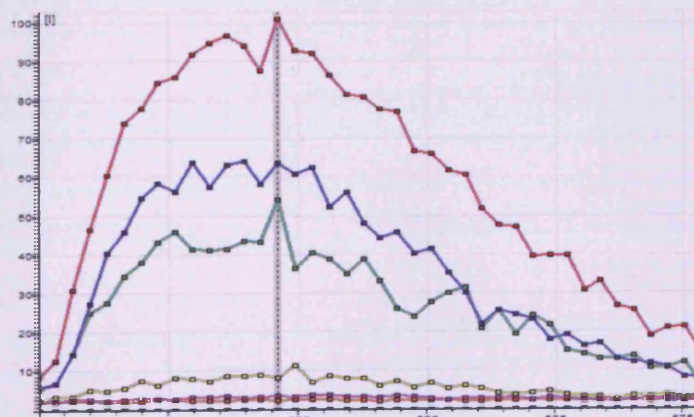
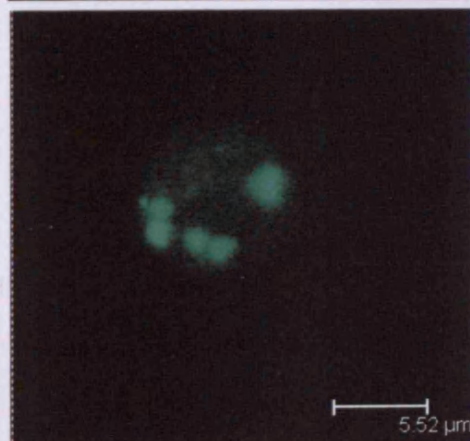
Fig. 8. 4. Autofluorescence spectra of brightly fluorescent inclusions within cytoplasm of *Spironucleus vortens* trophozoites excited at 458 nm. Lambda scans were performed by 10 nm increments from 470 nm to 680 nm on a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 548 Ar Ion laser at 61% output intensity. Fluorescence intensity was recorded as the mean amplitude of pixel intensity in selected regions of interest.

A



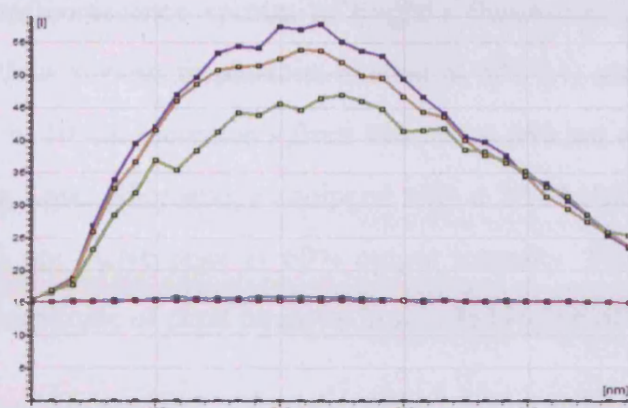
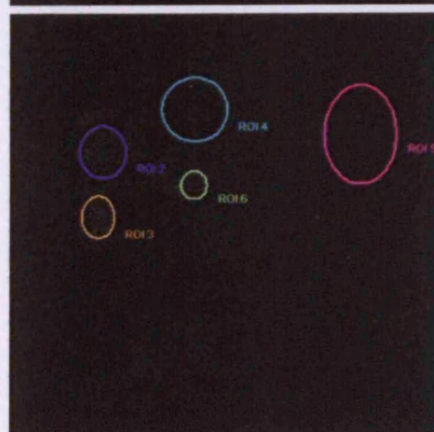
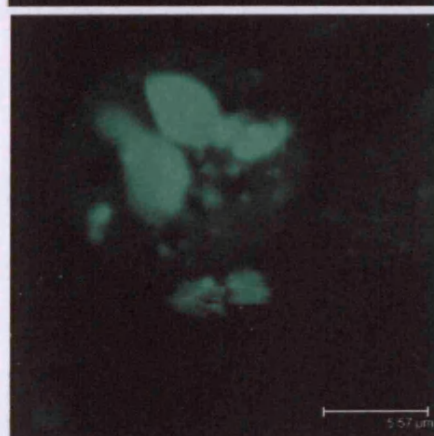
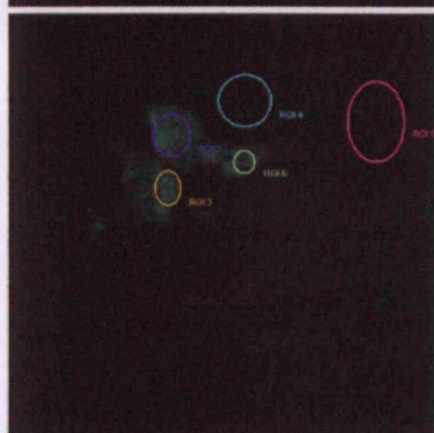
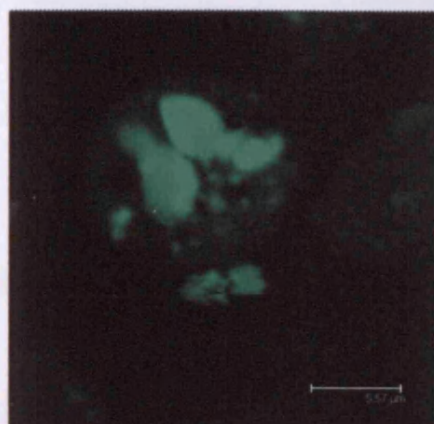
ROI	ROI 8	ROI 9	ROI 10	ROI 11	ROI 12	ROI 13	ROI 14
#Pixel(ROI)	523.00	397.00	438.00	5648.00	596.00	1579.00	785.00
Area	1.52 μm^2	1.15 μm^2	1.27 μm^2	16.43 μm^2	1.74 μm^2	4.59 μm^2	2.28 μm^2
Length	683.00 nm	683.00 nm	683.00 nm	683.00 nm	683.00 nm	683.00 nm	683.00 nm
Mean Amplitude	55.13	26.07	73.82	2.55	5.75	3.17	5.87
Max. Amplitude	89.98	45.99	133.92	6.50	10.59	7.39	9.62
Pos. Max. Ampl.	548.10 nm	548.10 nm	533.90 nm	470.00 nm	548.10 nm	470.00 nm	541.00 nm
Min. Amplitude	8.94	6.03	12.01	2.05	2.04	1.98	2.12
Pos. Min. Ampl.	675.90 nm	675.90 nm	675.90 nm	675.90 nm	668.80 nm	675.90 nm	668.80 nm
Average Deviation	21.43	10.57	34.24	0.45	2.08	0.79	2.10
Standard Deviation	24.95	12.44	39.59	0.87	2.41	1.11	2.36

B

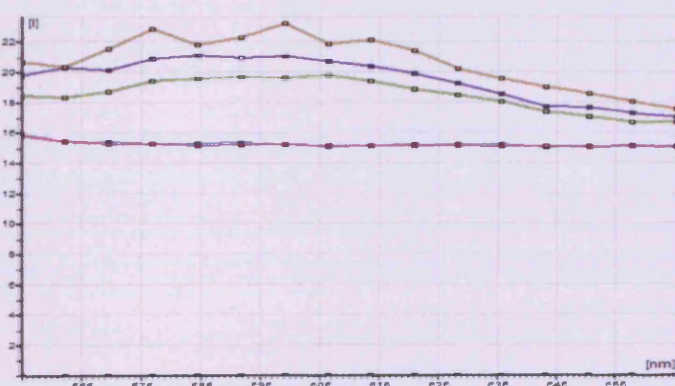


ROI	ROI 8	ROI 9	ROI 10	ROI 11	ROI 12	ROI 13	ROI 14
#Pixel(ROI)	523.00	397.00	438.00	5648.00	596.00	1579.00	785.00
Area	1.52 μm^2	1.15 μm^2	1.27 μm^2	16.43 μm^2	1.74 μm^2	4.59 μm^2	2.28 μm^2
Length	682.00 nm	682.00 nm	682.00 nm	682.00 nm	682.00 nm	682.00 nm	682.00 nm
Mean Amplitude	59.87	27.69	36.37	2.27	5.82	2.84	5.79
Max. Amplitude	102.90	55.36	65.34	2.67	13.18	3.85	11.00
Pos. Max. Ampl.	514.95 nm	514.95 nm	502.10 nm	469.97 nm	521.37 nm	534.22 nm	521.37 nm
Min. Amplitude	8.73	5.33	5.80	2.00	2.36	2.12	2.25
Pos. Min. Ampl.	425.00 nm	425.00 nm	425.00 nm	675.57 nm	425.00 nm	431.43 nm	425.00 nm
Average Deviation	25.55	11.34	18.59	0.12	2.00	0.39	1.95
Standard Deviation	28.98	13.30	20.63	0.16	2.39	0.47	2.29

Fig. 8. 5. Comparison between autofluorescence spectra of brightly fluorescent inclusions within cytoplasm of a *Spirotrunculus vortens* trophozoite excited at 458 (A) and 405 nm (B). Lambda scans were performed by 10 nm increments from 420 nm to 680 nm on a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 548 nm Ar Ion laser at 61% or a 405 nm laser at 64% output intensity. Fluorescence intensity was recorded as the mean amplitude of pixel intensity in selected region of interest.



ROI	ROI 2	ROI 3	ROI 4	ROI 5	ROI 6
#Pixel(ROI)	1641.00	929.00	2813.00	4729.00	491.00
Area	4.85 μm^2	2.75 μm^2	8.32 μm^2	13.99 μm^2	1.45 μm^2
Length	656.00 nm	656.00 nm	656.00 nm	656.00 nm	656.00 nm
Mean Amplitude	41.55	39.78	15.80	15.55	36.27
Max. Amplitude	59.07	55.05	16.20	15.74	47.93
Pos. Max. Ampl.	527.47 nm	519.43 nm	535.50 nm	535.50 nm	535.50 nm
Min. Amplitude	15.80	15.79	15.39	15.41	15.71
Pos. Min. Ampl.	415.00 nm	415.00 nm	647.97 nm	647.97 nm	415.00 nm
Average Deviation	11.22	10.39	0.22	0.08	7.73
Standard Deviation	13.34	12.18	0.25	0.10	9.48



ROI	ROI 2	ROI 3	ROI 4	ROI 5	ROI 6
#Pixel(ROI)	1641.00	929.00	2813.00	4729.00	491.00
Area	4.85 μm^2	2.75 μm^2	8.32 μm^2	13.99 μm^2	1.45 μm^2
Length	665.00 nm	665.00 nm	665.00 nm	665.00 nm	665.00 nm
Mean Amplitude	19.92	21.10	15.55	15.51	18.87
Max. Amplitude	21.52	23.69	16.17	16.14	20.18
Pos. Max. Ampl.	582.50 nm	596.25 nm	555.00 nm	555.00 nm	603.13 nm
Min. Amplitude	17.32	17.90	15.36	15.35	16.99
Pos. Min. Ampl.	658.13 nm	658.13 nm	637.50 nm	644.38 nm	658.13 nm
Average Deviation	1.25	1.47	0.12	0.12	0.90
Standard Deviation	1.47	1.77	0.19	0.19	1.11

A

B

Fig. 8. 6. Comparison between autofluorescence spectra of brightly fluorescent inclusions within the cytoplasm of a *Spironucleus vortens* trophozoite excited at 405 (A) and 543 nm (B). Lambda scans were performed in 10 nm increments from 420 nm to 680 nm on a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 405 laser at 45% or a 543 nm HeNe laser at 60% output intensity. Fluorescence intensity was recorded as the mean amplitude of pixel intensity in selected region of interest.

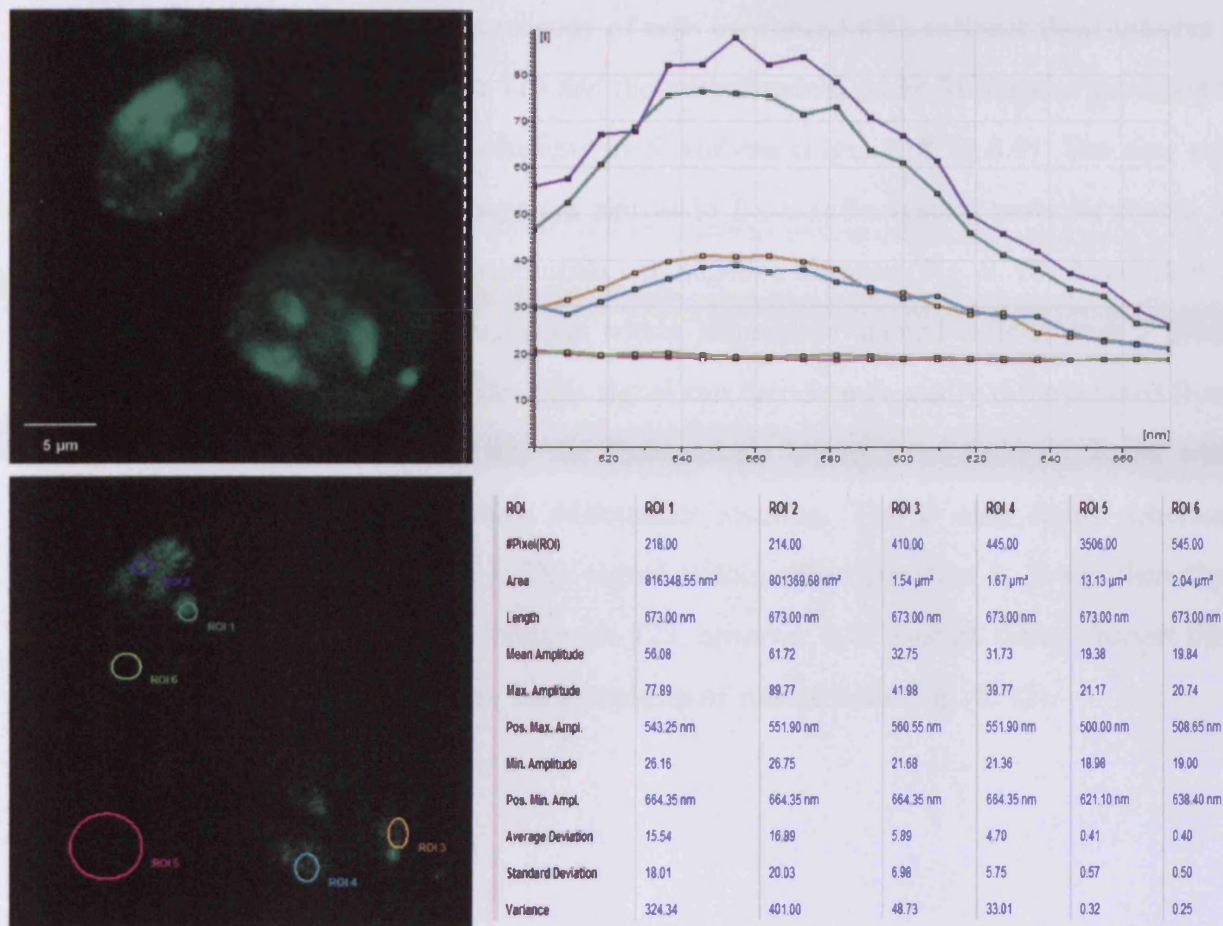


Fig. 8. 7. Autofluorescence spectra of brightly fluorescent inclusions within cytoplasm of *Spirotrunculus vortens* trophozoites excited at 488 nm. Lambda scans were performed by 10 nm increments from 470 nm to 680 nm on a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 588 Ar Ion laser at 29 % output intensity. Fluorescence intensity was recorded as the mean amplitude of pixel intensity in selected region of interest.

8. 4. 3 Confocal scanning laser microscopy of cells incubated with cationic fluorophores

The cationic fluorophore rhodamine 123 and the mitochondrial probe Mitotracker green stain numerous spherical cytoplasmic inclusions in *S. vortens* (Figs. 8. 8 & 8.9). The size and distribution of these stained inclusions are similar to the autofluorescent particles shown in Figure 8. 4. However comparison with unstained, negative samples (Fig. 8. 10) demonstrates that the signal from fluorescent inclusions within Mitotracker-stained cells is over 7 times stronger than that of autofluorescence. This signal can therefore be easily differentiated from autofluorescence, which confirms that the fluorescence signal from cells incubated with Mitotracker genuinely originates from Mitotracker staining. TMRE also stains spherical inclusions within cells (Fig. 8. 11). The signal within these particles is lower than that observed with Mitotracker green or rhodamine 123, however, it is over 35 times stronger than that of unstained, negative controls or the cytoplasm of stained cells (Fig. 8. 12).

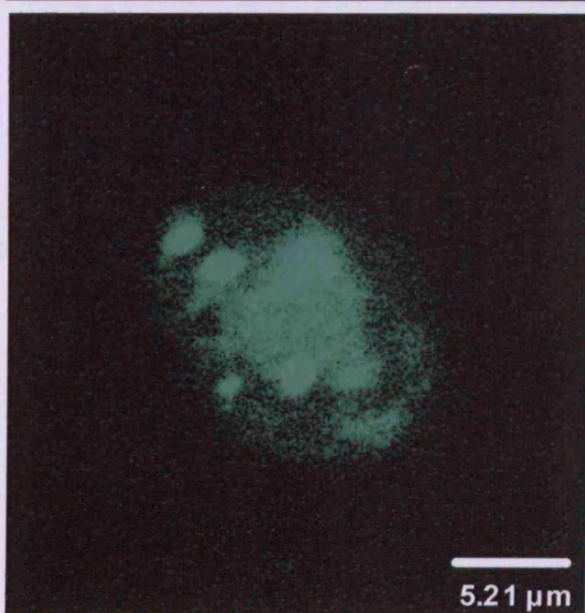
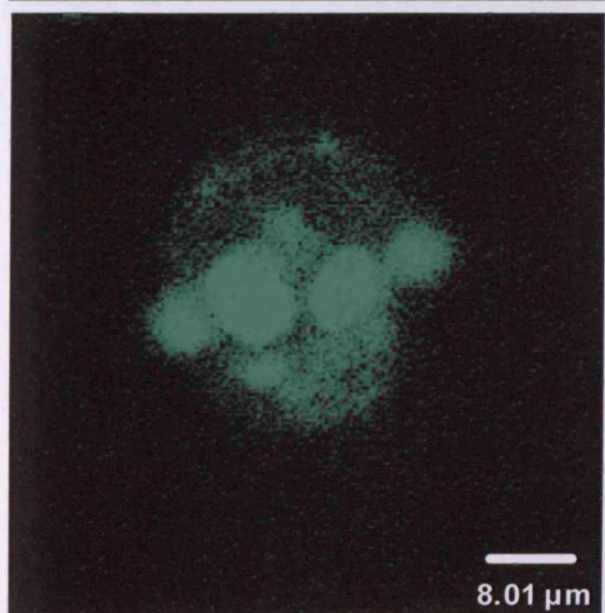
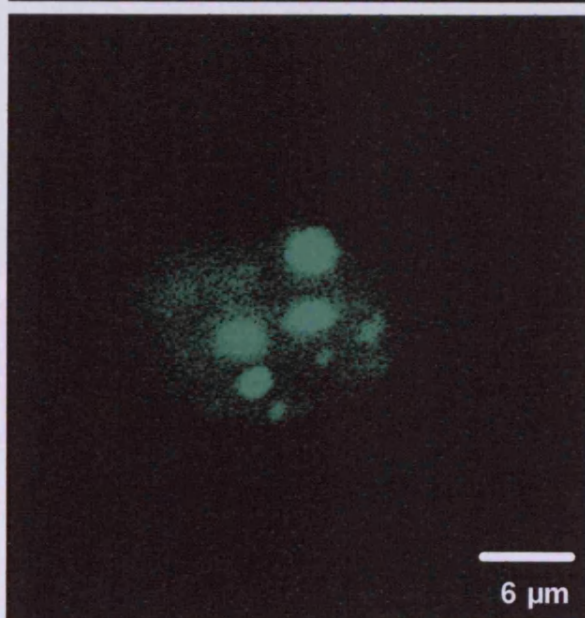
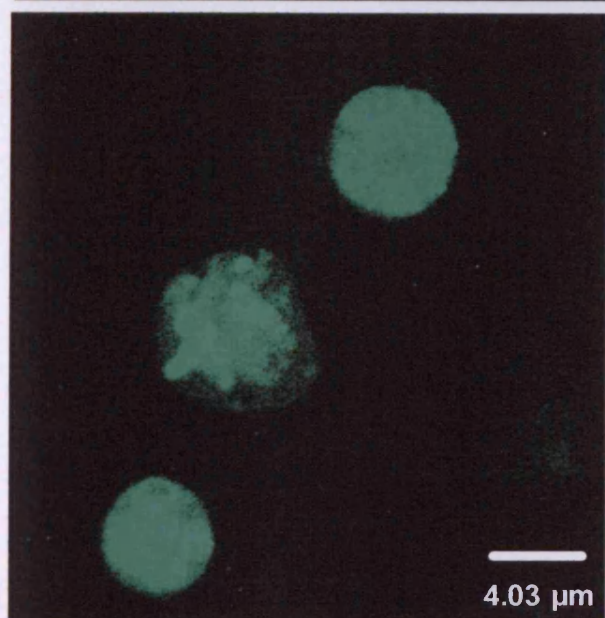
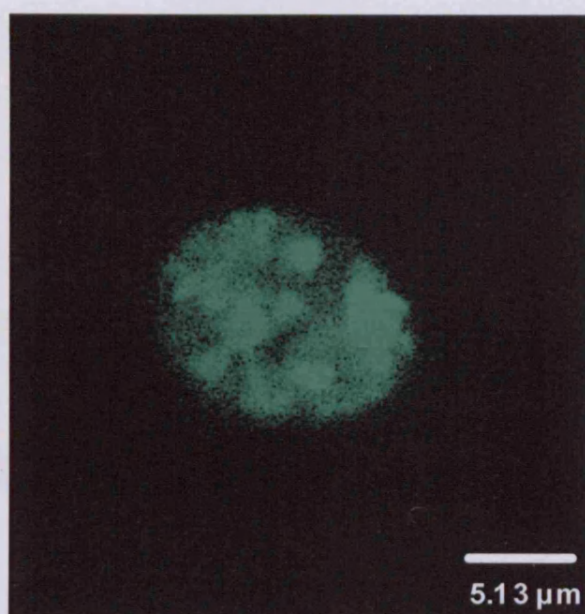
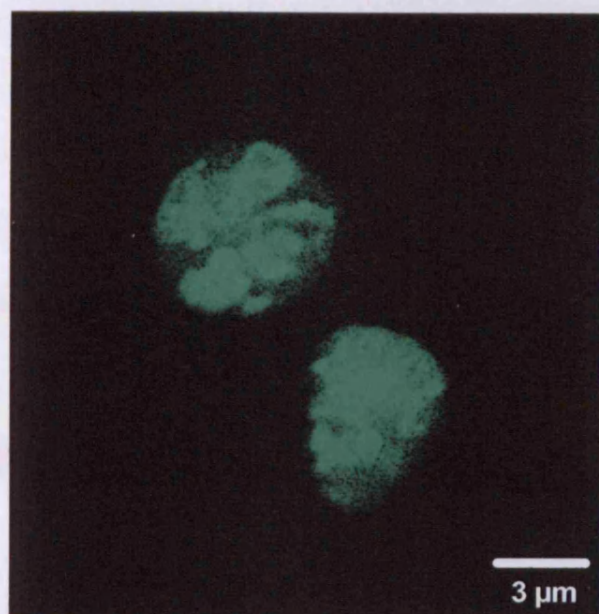


Fig. 8. 8. Three-dimensional maximum intensity projections of *Spironucleus vortens* trophozoites incubated with the cationic fluorophore rhodamine 123. Fluorescence was recorded at 520 nm on a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X63 objective and using a 588 Ar Ion laser at 20% output intensity.

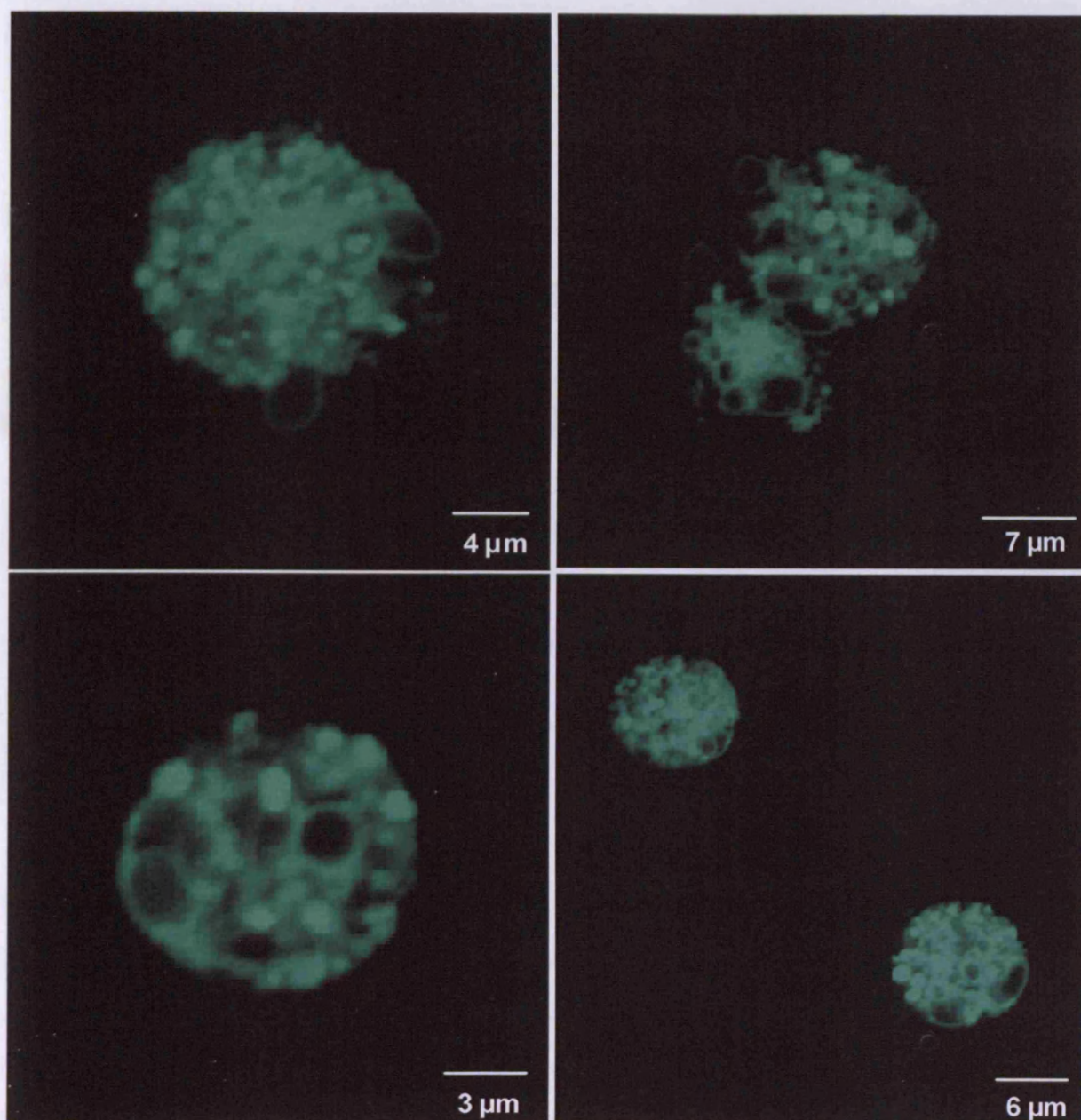


Fig. 8. 9. Three-dimensional maximum intensity projections of *Spiromucleus vortens* trophozoites incubated with the mitochondrial probe Mitotracker green. Fluorescence was recorded at 520 nm on a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 588 Ar Ion laser at 20% output intensity.

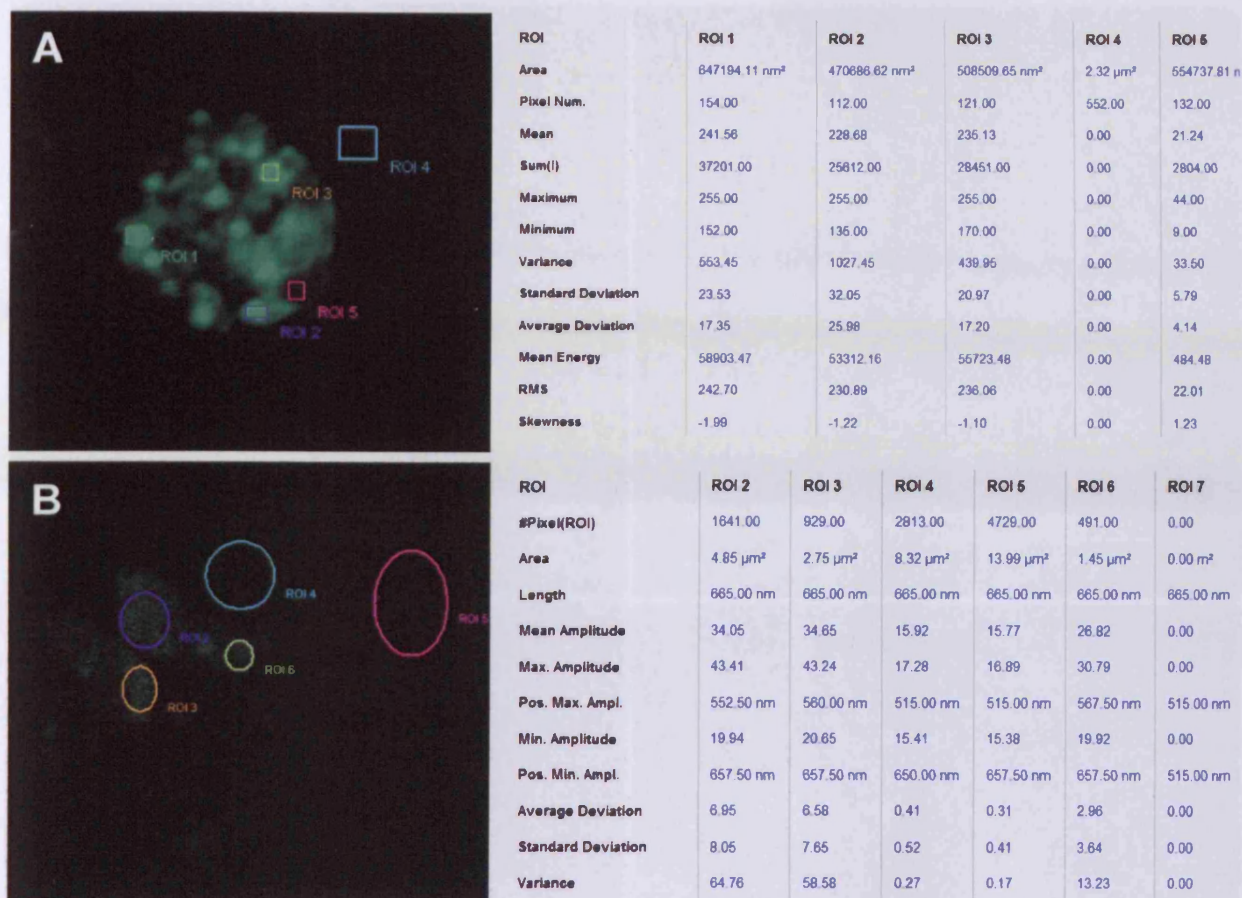


Fig. 8. 10. Comparison of fluorescence intensity within bright inclusions and cytoplasm of Mitotracter green-stained *Spirotrunculus vortens* trophozoites (A) and in unstained, control cells (B). Fluorescence intensity was recorded at 520 nm as the mean amplitude of pixel intensity in selected region of interest using a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and a 588 Ar Ion laser at 20% (A) or 29% output intensity (B).

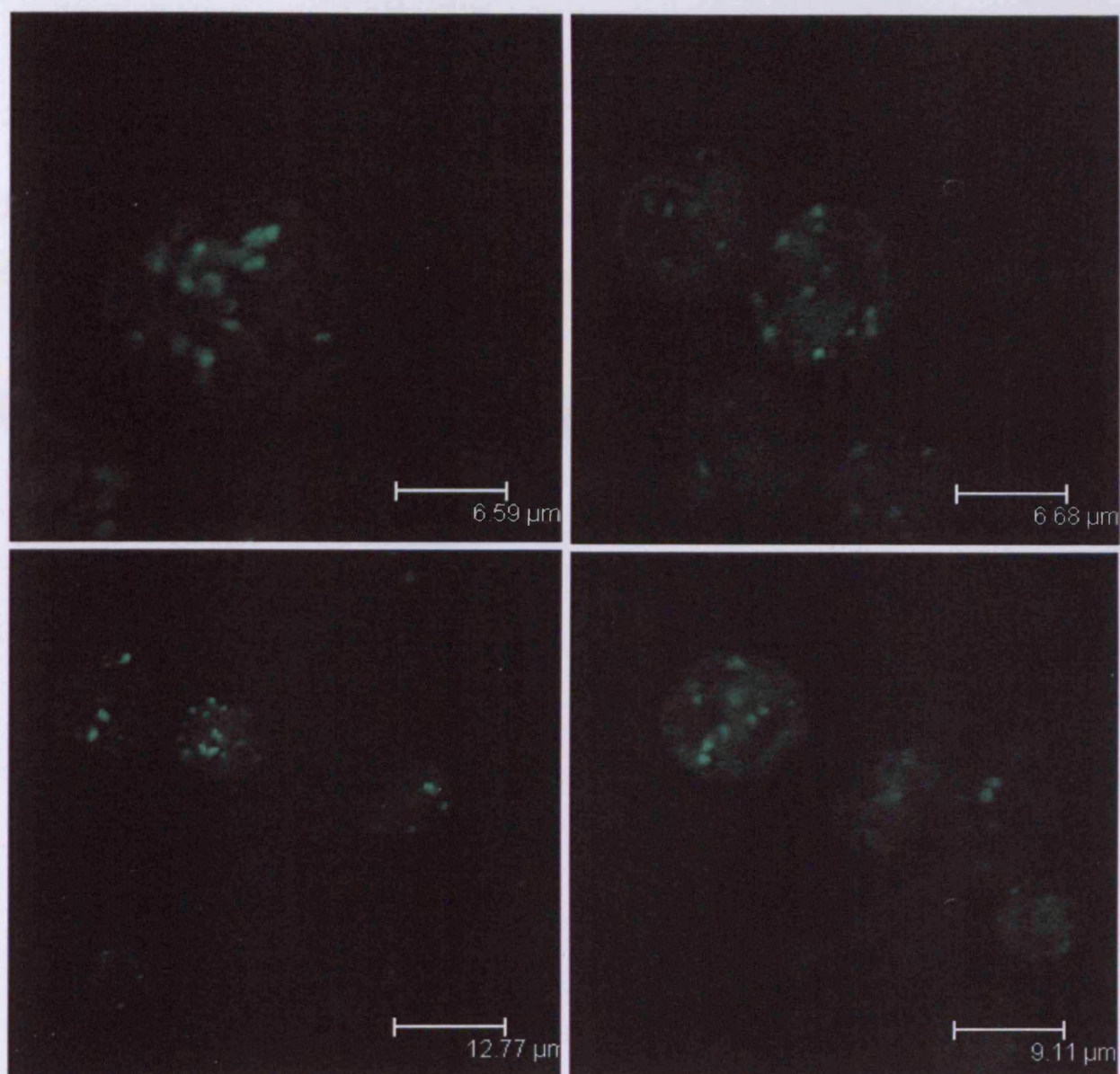


Fig. 8. 11. Confocal scanning laser micrographs of *Spironucleus vortens* trophozoites incubated with the cationic fluorophore tetra methyl rhodamine. Fluorescence was recorded at 585 nm on a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 543 HeNe laser at 60% output intensity.

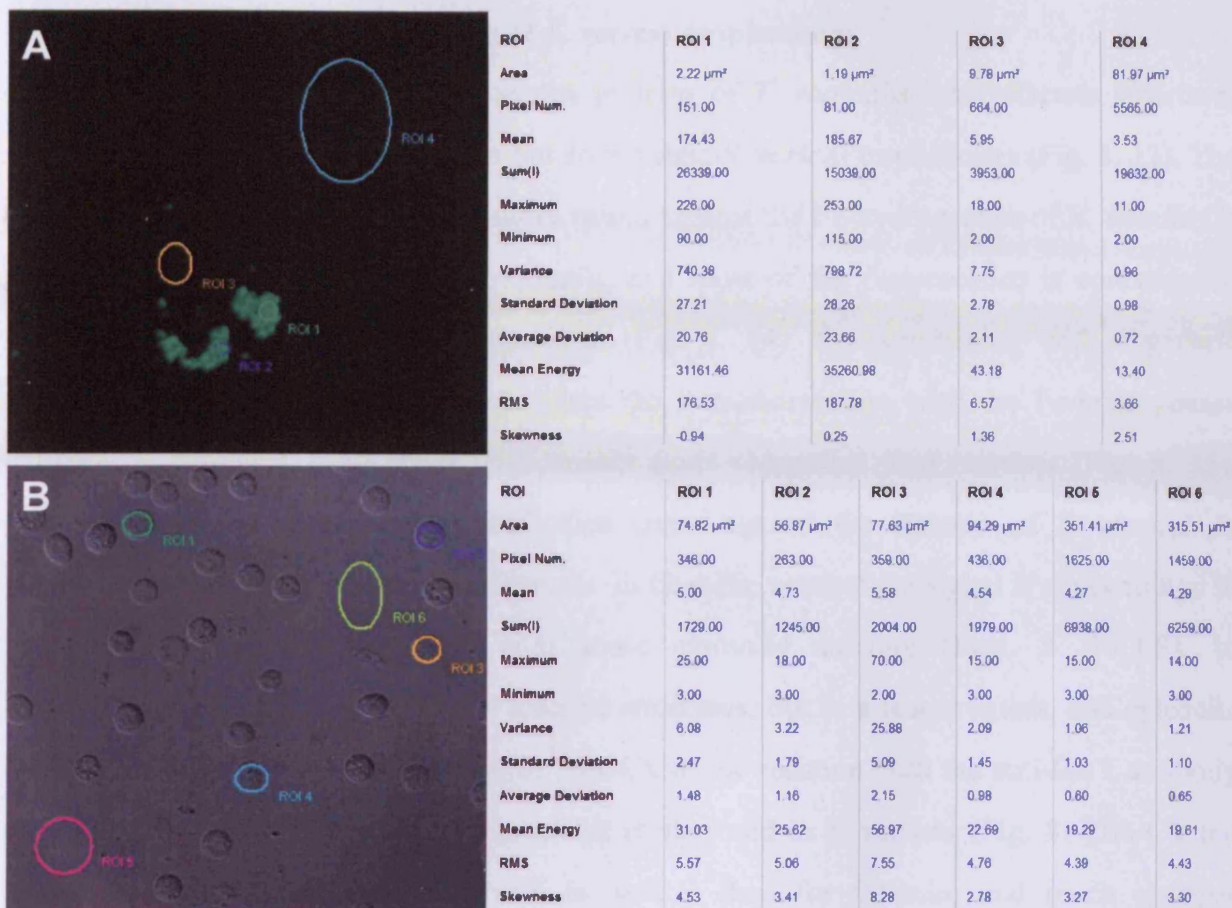


Fig. 8. 12. Comparison of fluorescence intensity within bright inclusions and cytoplasm of tetra methyl rhodamine-stained *Spironucleus vortens* trophozoites (A) and in unstained, control cells (B). Fluorescence intensity was recorded at 585 nm as the mean amplitude of pixel intensity in selected region of interest using a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and a 543 HeNe laser at 60% (A) or 65% (B) output intensity.

8. 4. 4 Immunofluorescent labelling of *S. vortens* trophozoites

Antibodies raised against hydrogenosomal proteins of *T. vaginalis* label discrete structures within the cytoplasm of this organism but do not stain *S. vortens* trophozoites (Fig. 8. 13). The immunostaining reaction using antibodies raised against the Fe-hydrogenase of *B. hominis* is positive in both *S. vortens* and *T. vaginalis*, and most of the fluorescence is concentrated within discrete particles in both organisms (Fig. 8. 14). Counterstaining with a generic membrane stain, shows that the area where the immunoreaction with the Fe-hydrogenase antibody is strongest, colocalises with membranous organelles in *S. vortens* (Fig. 8. 15). Immunofluorescent reaction with antibodies raised against the frataxin of *E. cuniculi* is positive in both *S. vortens* and *G. intestinalis*. In *Giardia*, most of the signal is concentrated in very small, spherical organelles, with some cytosolic staining (Figs. 8. 16D-F). In *Spiromucleus*, staining also occurs in discrete structures, but to a lesser extent, and cytosolic background staining is stronger (Figs. 8. 16A-C). In the reaction with the anti-Isu 1 antibody raised in yeast, labelling of discrete particles is observed in *S. vortens* (Fig. 8. 17A-C), but labelling of these individual structures is weaker than for frataxin, and much stronger background labelling occurs in the cytoplasm. As with frataxin, labelling of clear, discrete particles is also observed in *G. intestinalis* (Figs. 8. 17D-F). Strong staining occurs in *S. vortens* trophozoites incubated with an antibody raised against the ferredoxin of *E. cuniculi* (Fig. 8. 18). Labelling occurs uniformly at low levels throughout cytoplasm, and very strongly in one discrete area, near flagella pockets, in the anterior section of each cell.

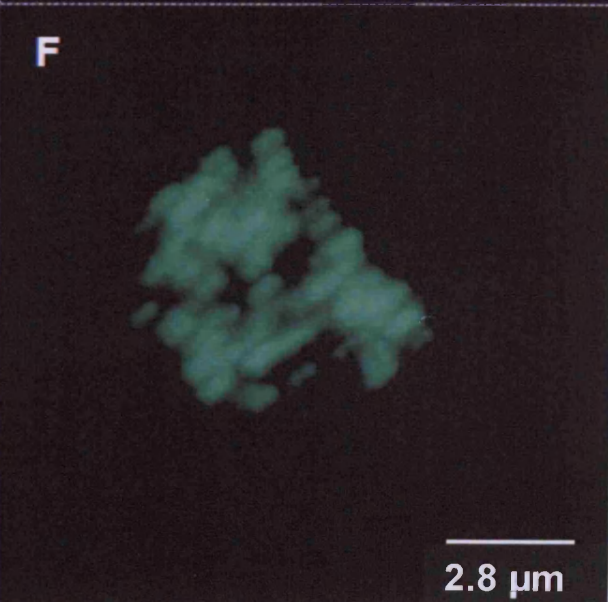
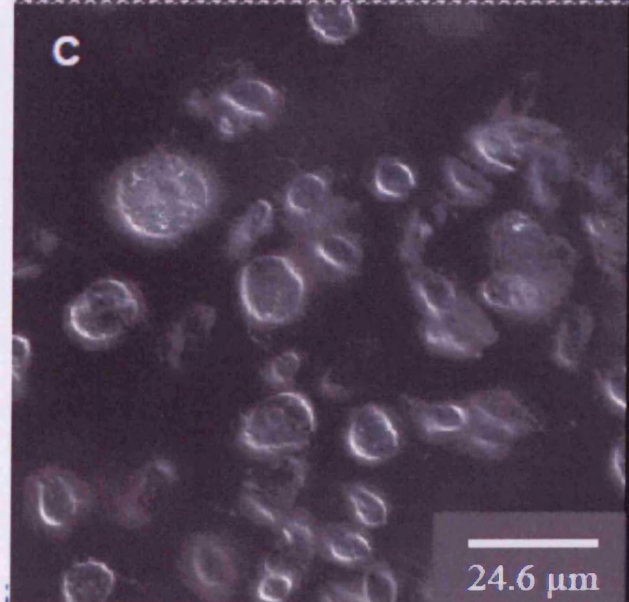
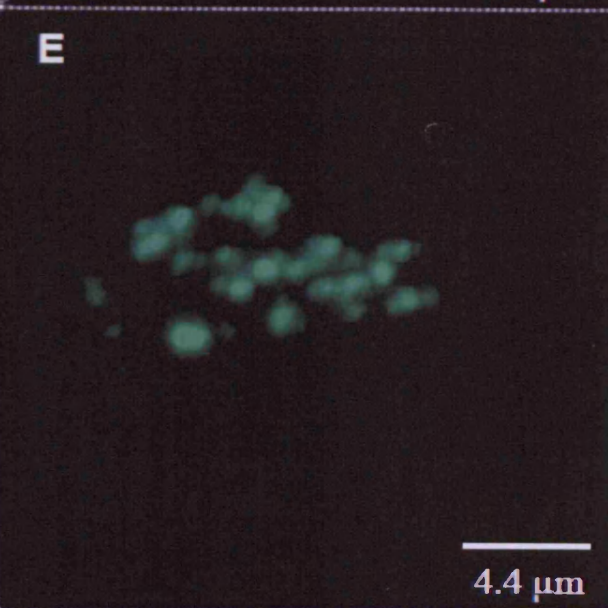
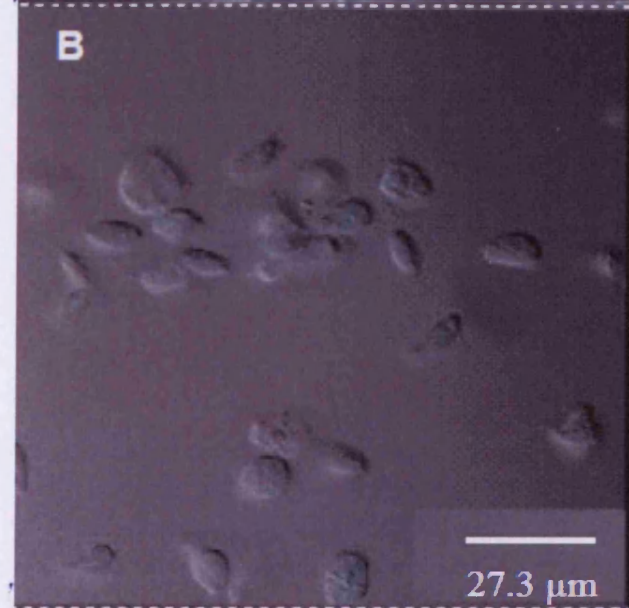
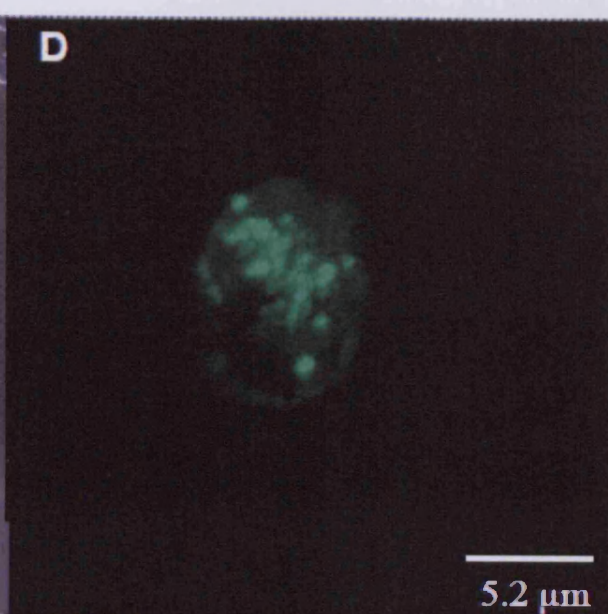
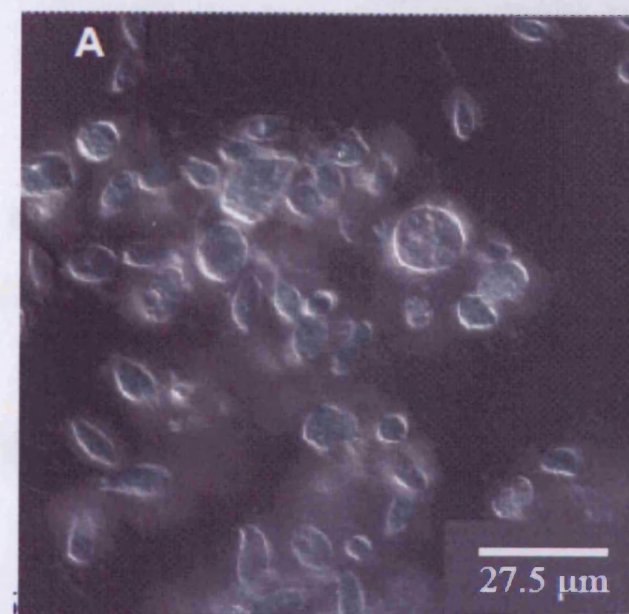


Fig. 8. 13. Three-dimensional maximum intensity projections of *Spironucleus vortens* incubated with monoclonal antibodies raised against the α -subunit of succinyl-CoA synthetase (A) and the β subunit of succinyl-CoA synthetase (B) of *Trichomonas vaginalis*; (C) and a polyclonal antibody raised against hydrogenosomal granular fraction of *T. vaginalis*; (D) anti- α subunit of succinyl-CoA synthetase; (E), anti- β subunit of succinyl-CoA synthetase and (F) polyclonal anti-granule antibody. A, B and C are overlays of three-dimensional maximum intensity projections and their corresponding transmitted light images; photographs D, E and F are three-dimensional maximum intensity projections only. Preparations were viewed at 525 nm under a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X63 objective and using a 488 Ar Ion laser at 20% output intensity. *T. vaginalis* was used for positive controls

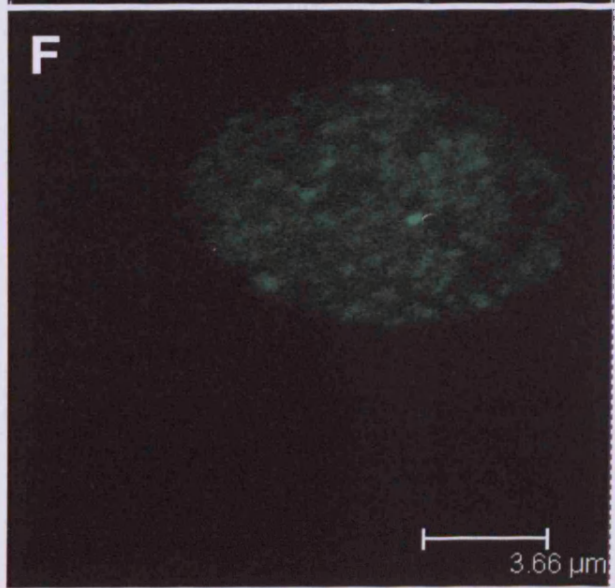
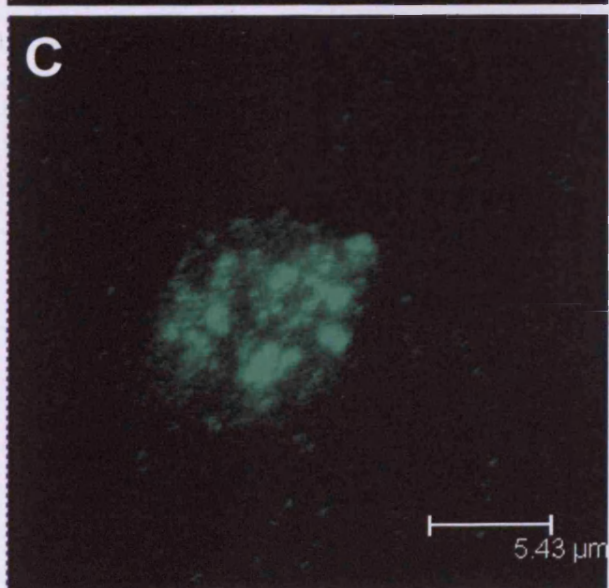
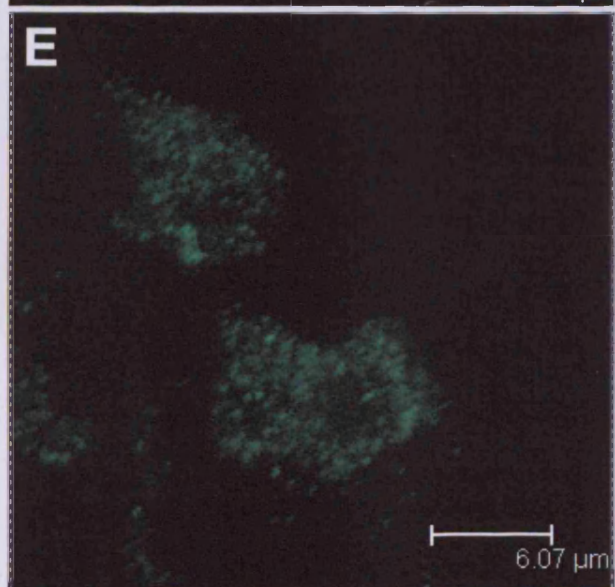
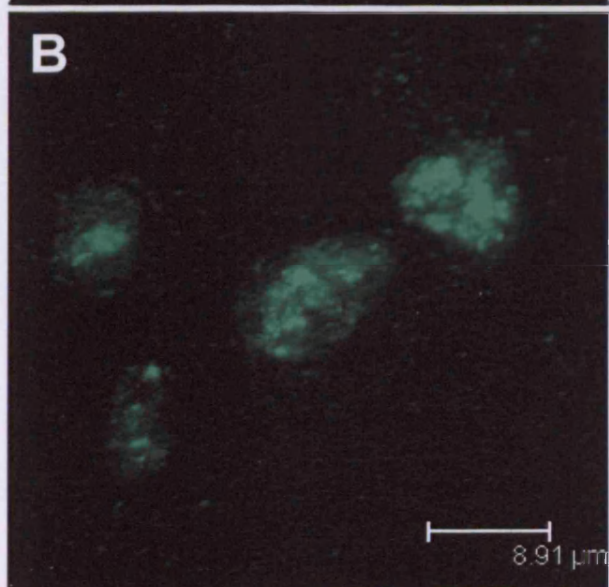
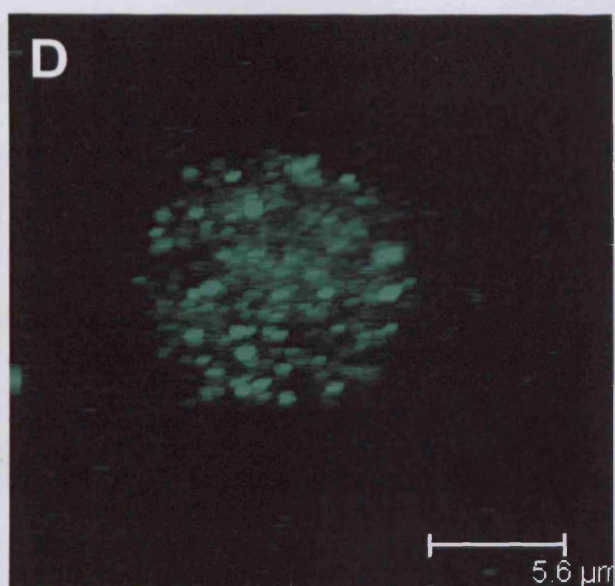
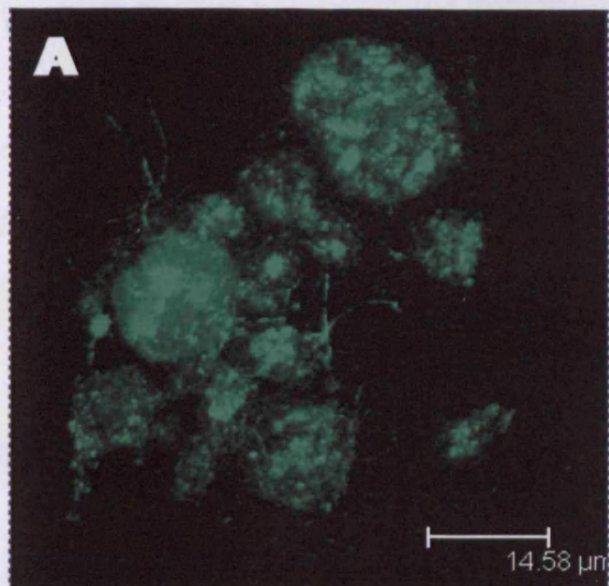


Fig. 8. 14. Three-dimensional maximum intensity projections of *Spirotrunculus vortens* (A-C) and *Trichomonas vaginalis* (D-F) trophozoites incubated with an FITC-labelled antibody raised against the Fe-hydrogenase of *Blastocystis hominis*. Preparations were viewed at 525 nm under a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 488 Ar Ion laser at 25% (A-C) and 35% output intensity (D-F).

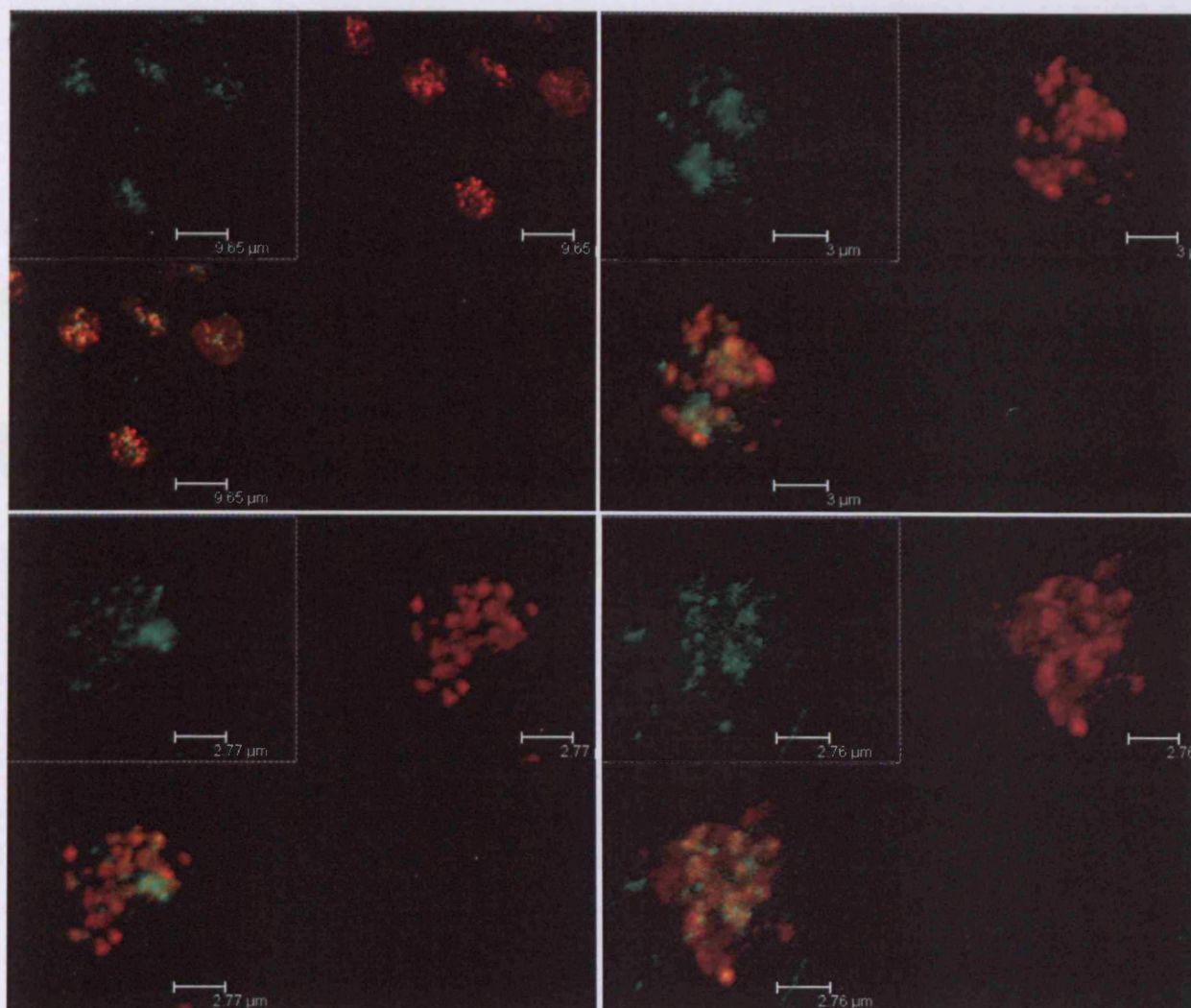


Fig. 8. 15. Confocal scanning laser micrographs of *Spironucleus vortens* trophozoites incubated with an FITC-labelled antibody raised against the Fe-hydrogenase of *Blastocystis hominis* (green) and counterstained with a lipophilic styryl dye, FM 1-43 (red). Preparations were viewed at 525 nm (green) and 600 nm (red) under a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 488 Ar Ion laser at 26% output intensity.

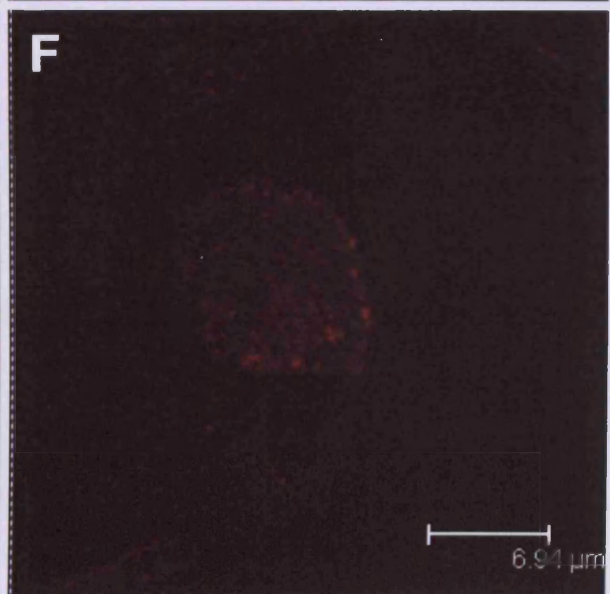
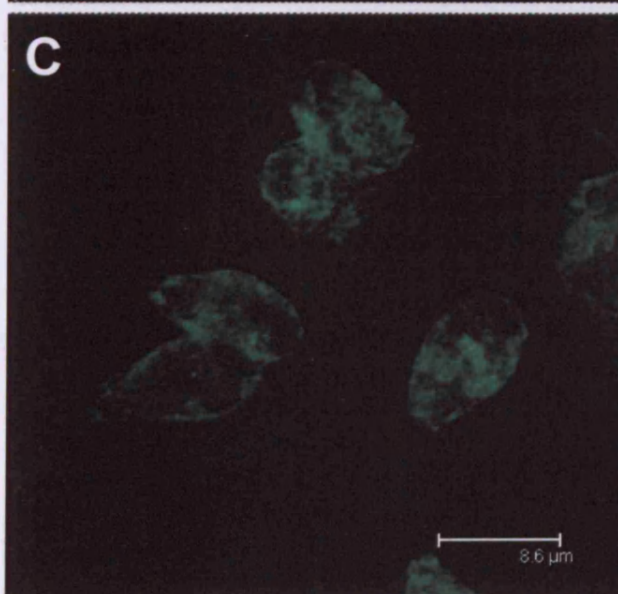
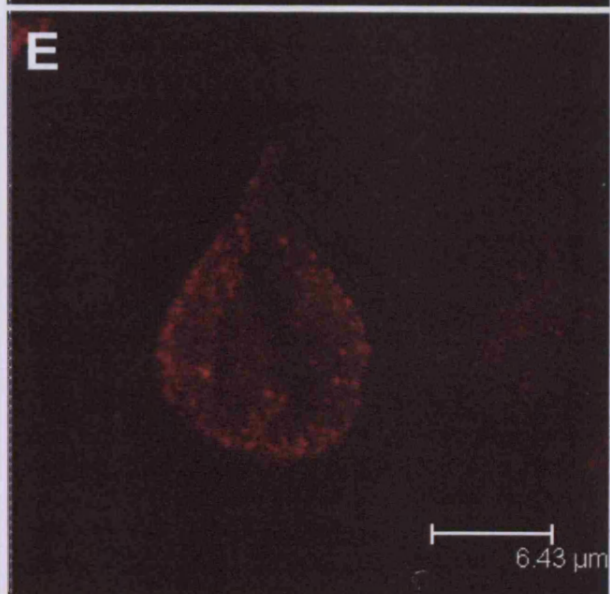
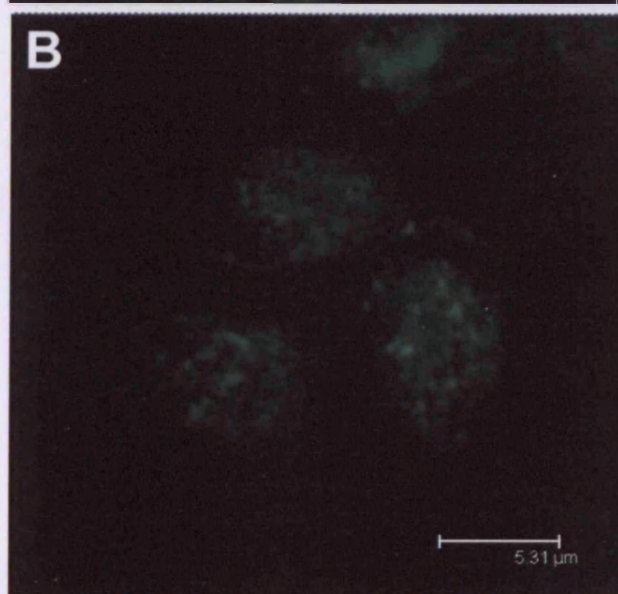
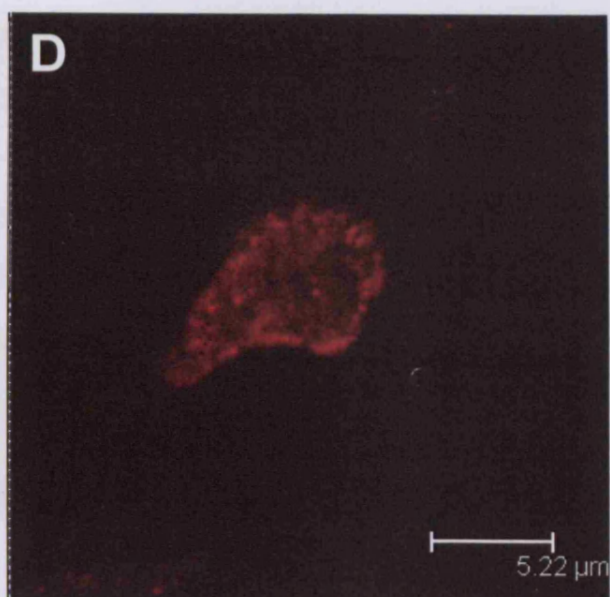
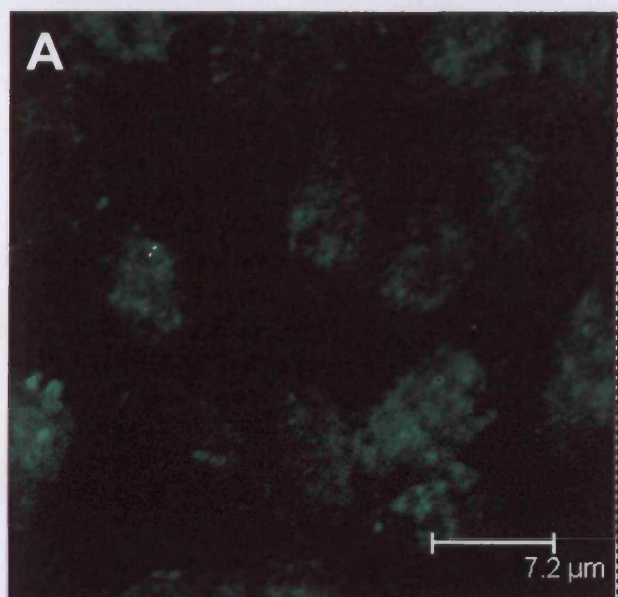


Fig. 8. 16. Confocal scanning laser micrographs of *Spironucleus vortens* (A-C) and *Giardia intestinalis* (D-F) incubated with an antibody raised against the frataxin of *Encephalitozoon cuniculi* and conjugated with an Alexa 488 (A-C) or an Alexa 594 (C-E)-labelled secondary antibody. Preparations were viewed at 520 or 620 nm under a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 488 nm Ar Ion laser at 18% (A-C) or a 594 nm HeNe laser at 49% output intensity (D-F). Preparation was exposed to UV light prior to antibody labelling to remove all background autofluorescence.

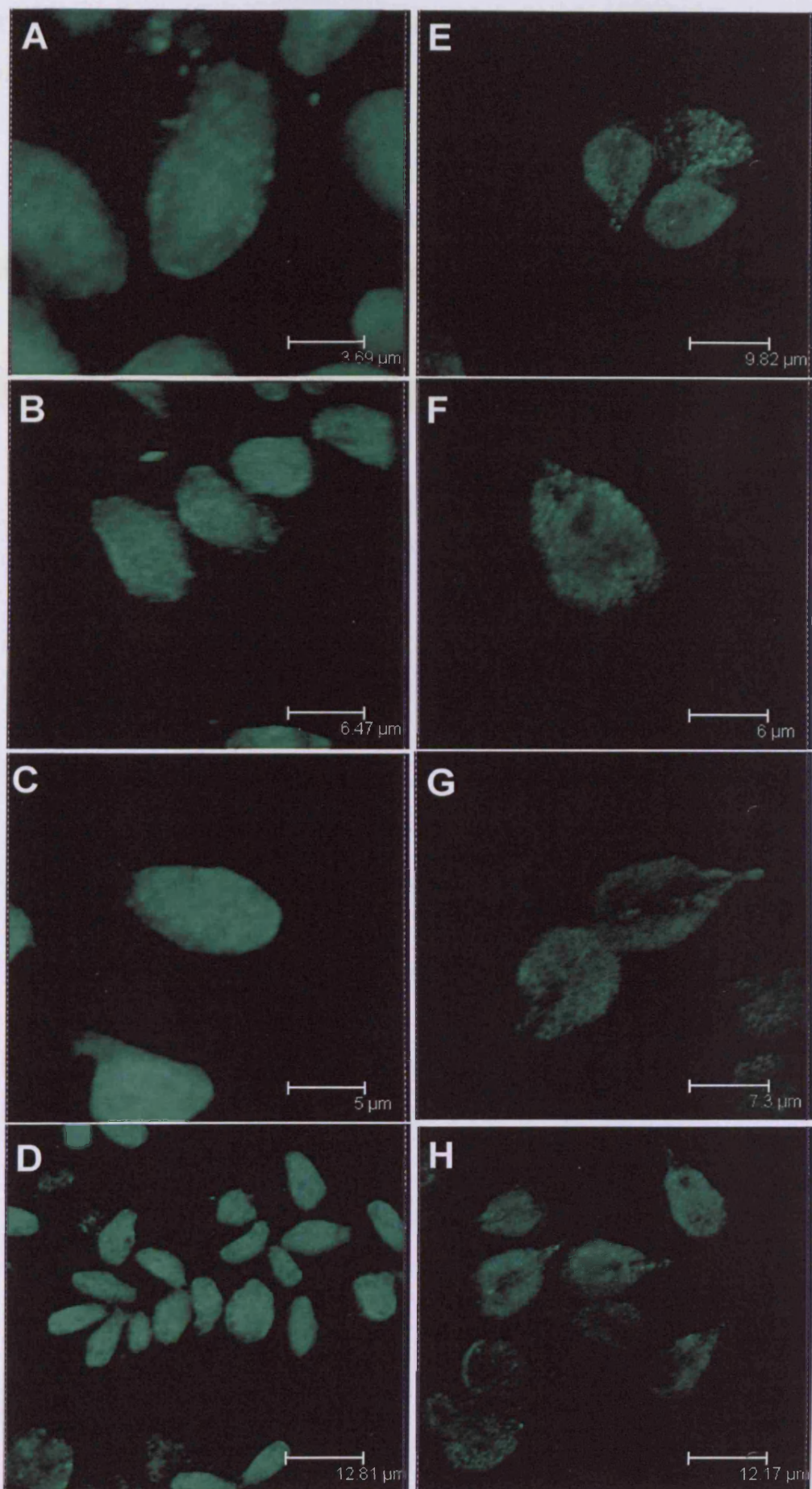


Fig. 8. 17. Confocal scanning laser micrographs of *Spironucleus vortens* (A-D) and *Giardia intestinalis* (E-H) trophozoites incubated with an Alexa 488-labelled antibody raised against the Isu1 of *Saccharomyces cerevisiae*. Preparations were viewed at 520 nm under a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 488 Ar Ion laser at 25% (A-D) or 29% (E-H). Preparation was exposed to UV light prior to antibody labelling to remove all background autofluorescence.

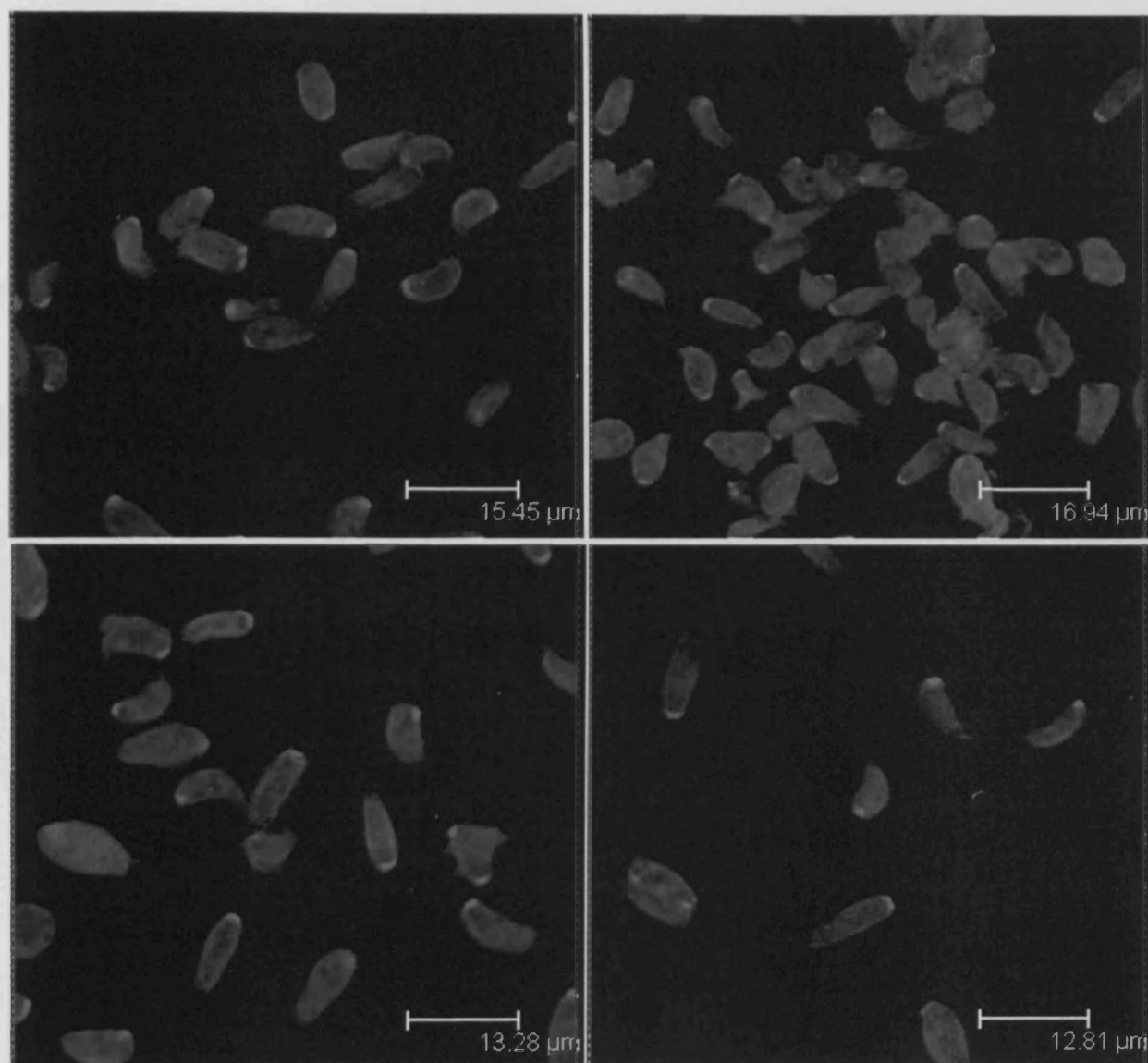


Fig. 8. 18. Confocal scanning laser micrographs of *Spironucleus vortens* trophozoites incubated with a FITC-labelled antibody raised against the ferredoxin of *Encephalitozoon cuniculi*. Preparations were viewed at 520 nm under a Leica TCS SP2 AOBS Confocal Scanning Laser Microscope equipped with a X100 objective and using a 488 Ar Ion laser at 25%. Preparation was exposed to UV light prior to antibody labelling to remove all background autofluorescence.

8. 5 DISCUSSION

1. Presence of putative mitosomes

Snap-freeze scanning electron microscopy reveals that *Spironucleus vortens* contains structures strongly resembling the mitosomes of *Giardia* and microsporidians (Tovar, Fischer et al. 1999; Tovar, Leon-Avila et al. 2003; Vavra 2005; Aguilera, Barry et al. 2008; Goldberg, Molik et al. 2008). These organelles in *S. vortens* are not made evident by standard TEM, but appear in micrographs of snap-freeze, cryo-sectioned trophozoites as small (100 to 150 nm), oval shaped structures with the clear double membrane distinctive of mitosomes. The very small size of these structures allows them to be easily distinguished from flagellar sections, which are significantly larger (>290 nm). Mitosomes of anaerobic eukaryotes are believed to be degenerate mitochondria (Tovar, Fischer et al. 1999; Dyllal and Johnson 2000; Vivares, Gouy et al. 2002; Tovar, Leon-Avila et al. 2003; Leon-Avila and Tovar 2004; Burri, Williams et al. 2006), which have lost their oxidative phosphorylation-dependant ATP generation functions in the evolutionary adaptation to an anaerobic lifestyle and have retained only the most essential functions of mitochondria, such as cellular iron metabolism and maturation of Fe-S cluster scaffolds (Katinka, Duprat et al. 2001; Tachezy, Sanchez et al. 2001; LaGier, Tachezy et al. 2003; Tovar, Leon-Avila et al. 2003; Abrahamsen, Templeton et al. 2004; Lill and Muhlenhoff 2005; van der Giezen, Leon-Avila et al. 2005; Goldberg, Molik et al. 2008; Lill and Muhlenhoff 2008; Lill 2009). The reasons why these functions have remained compartmentalised in the mitosome instead of becoming entirely cytosolic are unclear (Lill 2009). However, this organelle is present in phylogenetically distant lineages, which indicates convergent evolution (van der Giezen and Tovar 2005a&b), and therefore suggests an essential function for such compartmentalisation.

Antibodies raised against frataxin and Isu1, proteins involved in Fe-S cluster synthesis, in the mitosome of *Encephalitozoon cuniculi* and the mitochondria of yeast (Muhlenhoff, Richhardt et al. 2002; Gerber, Muhlenhoff et al. 2003), respectively, labelled *S. vortens*, as well as control, *G. intestinalis* trophozoites. In *Giardia*, these proteins seem to localize mostly in mitosomes, with low levels of cytosolic staining. In *S. vortens*, staining also occurs in small, discrete organelles, which we therefore propose to be mitosomes. The

number of these putative mitochondria in *S. vortens* is considerably lower than in *G. intestinalis*, and significant labelling also occurs in the cytoplasm. UV-bleaching and careful comparison with negative controls ensures that this pattern of fluorescence is not due to autofluorescence or non-specific labelling by the secondary antibody, but reflects the actual distribution of Isu1 and frataxin, which suggests that in *S. vortens*, significant amounts of Fe-S cluster synthesis proteins are present, both in the cytosol and in putative mitochondria. In turn, this indicates that in this organism, the functional site for Fe-S cluster assembly may not be solely mitochondrial. While this finding is atypical of mitochondrial organisms, it is not without precedent. Indeed, although in most organisms studied so far, including the microsporidian *E. cuniculi*, the complete set of Fe-S cluster synthesis proteins is contained in the mitochondrion, large pools of cytosolic Isu1 and frataxin were also recently reported in the microsporidian *Trachipleistophora hominis* (Goldberg, Molik et al. 2008). This confirms that even within the same phylogenetic branch, great heterogeneity exists in mitochondrial function. Further characterisation of putative mitochondria of *S. vortens* would require immunolocalisation of other components of the Fe-S cluster synthesis machinery, or other typical mitochondrial proteins, such as Hps70.

Interestingly, ferredoxin, a ubiquitous Fe-S redox carrier, also involved in Fe-S cluster synthesis (Lange, Kaut et al. 2000) is present in the cytosol of *S. vortens*, but does not appear to be concentrated within small, discrete organelles, as was observed for frataxin and Isu1. Instead, large accumulations of frataxin are seen at the anterior region of the organism. The function of such an atypical accumulation of ferredoxin is unclear. It is possible that, like the siderophores of some fungi (Matzanke, Bill et al. 1987), this enzyme may serve as a form of intracellular iron storage, and further investigations in iron-starved cells should be conducted.

2. Presence of putative hydrogenosomes

Both snap-freeze and standard TEM indicate that in addition to putative mitochondria, *S. vortens* trophozoites contain larger, double membrane and electron-dense core structures. Typically, the only large, double-membrane structures which appear so dense in electron micrographs are either hydrogenosomes or intracellular bacteria. However, there is no evidence of bacterial cell walls on these structures, and the *S. vortens* cultures used in the

current study were carefully monitored and kept free of bacterial contaminants (see Chapter 2). Moreover, fluorescent *in situ* hybridisation using 16S rDNA probes showed that trophozoites contained no intracellular bacteria (see Chapter 3), which renders the possibility of a bacterial origin for these electron-dense structures unlikely. Moreover, standard TEM shows that these structures morphologically resemble the hydrogenosomes of control *T. vaginalis* cells fixed and sectioned under identical conditions, and organelles in both organisms exhibit intricate membrane folds. This further advances the hypothesis of the hydrogenosomal nature of these particles.

Confocal scanning microscopy of unstained *S. vortens* trophozoites indicates that their cytoplasm contains brightly autofluorescent, spherical particles. Analysis of the autofluorescence spectra of these particles reveals that optimal absorption is obtained around 458 nm and emission occurs at around 530 nm. This spectrum is characteristic of the autofluorescence of oxidised FMN, FAD and riboflavins of mitochondria (Chance and Williams 1956; Aubin 1979; Benson, Meyer et al. 1979; Billinton and Knight 2001; Rocheleau, Head et al. 2003) and the high flavin concentration required to achieve such a strong autofluorescent signal indicates that these particles are almost certainly redox organelles.

The fluorescence spectrum of mitochondrial flavins has been extensively studied, both *in situ* and in purified solutions, as these molecules provide a powerful, non-invasive tool for the investigation of mitochondrial and hydrogenosomal function (Chance and Williams 1956; Galeotti, Van Rossum et al. 1970; Galeotti, van Rossum et al. 1970; Aubin 1979; Eng, Lynch et al. 1989; Heintzelman, Lotan et al. 2000; Billinton and Knight 2001; Rocheleau, Head et al. 2004; Yang, Li et al. 2008; Lloyd, D. and Wadley, R. unpublished data). Indeed, oxidised riboflavin, flavin coenzymes and flavoproteins from mitochondria were shown to be excitable at 460 nm and to emit at 530 nm, whereas, reduced, nicotine-based coenzymes (NAD(P)H) are optimally excited at 360 nm, with emission between 440 and 460 nm (Billinton and Knight 2001; Rocheleau, Head et al. 2003; Chance 2004; Chance, Nioka et al. 2005). Comparing fluorescence spectra at 450 and 530 nm therefore provides very precise information on the redox state of mitochondria in live cells. The strong flavin-based fluorescence observed in the putative redox organelles of *S. vortens* indicates that cells were

in a highly oxidised state, which is in accordance with the experimental conditions, whereby cells were mounted on slides for extended period of times, under atmospheric O₂ tensions.

Interestingly, upon excitation at 488 nm, the putative redox organelles produce a fluorescent signal at 555 nm. This red-shifted emission peak is typical of bound flavins, such as flavoproteins (Benson, Meyer et al. 1979; Billinton and Knight 2001). Fluorescence of flavins is generally strongly quenched when they are bound to their cofactors, and to date, only two types of flavoproteins, lipoamide dehydrogenase (LipDH) and electron transfer flavoprotein (ETF), are known to produce a fluorescent signal strong enough to be detected. Unlike ETF and free flavins, which are optimally excited at 460 nm, LipDH, was shown to be preferably excited at 488 nm (Rocheleau, Head et al. 2003). The 555 nm emission peak observed upon excitation at 488 nm may therefore indicate the presence of a LipDH protein in the putative redox organelles of *S. vortens*. As LipDH is a typical mitochondrial protein, also found in the hydrogenosome of *T. vaginalis* (Mukherjee, Brown et al. 2006), its presence would also substantiate the hypothesis of a redox nature for these autofluorescent, flavin-rich particles.

Incubation of *S. vortens* trophozoites with cationic fluorophores, such as rhodamine 123, TMRE or Mitotracker green, resulted in the staining of spherical organelles, whose size and distribution was identical to that of the FAD-rich, autofluorescent particles. Due to their positive charge, rhodamine 123 and TMRE accumulate on the negatively-charged side of membranes and have therefore been used extensively in the labelling of redox organelles such as mitochondria and hydrogenosomes (Chazotte 2006). Their labelling of spherical structures in *S. vortens* thus indicates the presence of organelles with a membrane potential. This, combined with the detection of flavin-packed organelles of similar size, constitutes strong evidence for the presence of mitochondria or hydrogenosome-like redox organelles in this organism. This conclusion is corroborated by the identical staining pattern obtained using Mitotracker green, a cationic probe which has been specifically designed for the labelling of mitochondria, and is retained by the organelles, even after loss of membrane potential (Chazotte 2006). This allows localisation of mitochondria and redox organelles in dead or dying cells. This property has been particularly useful in the imaging of *S. vortens*, as live

trophozoites of this highly motile organism are extremely difficult to immobilise, and imaging of redox organelles in live cells can be challenging when using regular cationic fluorophores.

Immunoconfocal microscopy using antibodies raised against hydrogenosomal proteins of *T. vaginalis* did not label *S. vortens*, but such a negative result may be attributed to the lack of cross-reactivity between proteins in organelles of organisms belonging to rather distant lineages, and thus does not refute the presence of hydrogenosome-like organelles in *S. vortens*. Moreover, the presence of hydrogenosomes in *S. vortens* is reasserted by the pattern of localisation of an Fe-Fe hydrogenase, a key hydrogenosomal enzyme (Lindmark and Muller 1973; Bui and Johnson 1996; Davidson, van der Giezen et al. 2002; Voncken, Boxma et al. 2002; Stechmann, Hamblin et al. 2008). Indeed, immunolocalisation of an antibody raised against the Fe-Fe hydrogenase of *Blastocystis hominis* (Lantsman, Tan et al. 2008) shows that this hydrogenase is present in low concentrations in the cytoplasm of *S. vortens* trophozoites, but concentrates mostly in large organelles, which appear to colocalise with spherical, membranous structures. Such discrete localisation of the Fe-Fe hydrogenase antibody is similarly observed in control samples of *T. vaginalis* cells, in which hydrogenase activity is known to be essentially hydrogenosomal (Lindmark and Muller 1973; Bui and Johnson 1996)

Along with the TEM and autofluorescence data, as well as the observed pattern of accumulation of cationic fluorophores, this constitutes extremely strong evidence for the presence of hydrogenosome-like, redox organelles in *S. vortens*. However, future work, including immunolocalisation of the Fe-Fe hydrogenase in TEM sections, is required to further validate this claim.

3. Evolutionary outlook

Isu1 and frataxin do not appear to label putative hydrogenosomes in *S. vortens*, which suggests that in this organism, the two main functions typically assigned to hydrogenosomes, namely hydrogen generation and maturation of Fe-S clusters, may be split into two morphologically distinct organelles. This lack of labelling of the larger putative hydrogenome is, of course, not conclusive evidence of the lack of an Fe-S cluster assembly function in these putative hydrogenosomes. Indeed, the observed presence of large cytosolic pools of both

proteins may enable functional Fe-S maturation within these organelles. Immunolocalisation of other components of the Fe-S maturation machinery should therefore be performed to further investigate this hypothesis.

Nevertheless, it remains that from an evolutionary point of view, the coexistence of mitosomal and hydrogenosomal-types of organelles in one organism is intriguing, and several explanations that could justify the presence of both organelles exist. The first possibility is that both organelles have derived from the same protomitochondrial or protohydrogenosomal endosymbiont and subsequently diverged functionally. In this scenario, the evolutionary drive for such functional segregation of both organelles is difficult to understand; however, as parasites sometimes encounter very atypical selective pressures, this theory cannot be dismissed. For instance, some parasites can have incredibly wasteful metabolism, and metabolism in bloodstream forms of trypanosomes, aerobic glycolysis, is known to be highly inefficient (Bakker, Mensonides et al. 2000; Bakker, Westerhoff et al. 2000), an evolutionary trait, which would seem counterproductive for most free-living organisms, but appears to be a successful survival strategy for this parasite. In the intestinal tract of its host, *S. vortens* may be exposed to fluctuating O₂ tensions (Pogrud and Steggerda 1948a&b; Smith and Morton 2001), and it is not impossible that it has evolved to express its hydrogenosomes exclusively during anaerobic conditions, only retaining a more reduced, mitosome-like form of the organelle when more O₂ is present. One may even speculate that the putative mitosomes and hydrogenosomes observed in the current study are simply different morphological expressions of the same organelle. Indeed, mitochondria are known to exhibit great morphological and functional plasticity, depending on life-cycle, nutrient availability (Giffin, McCann et al. 1986; Michelotti and Hajduk 1987), or oxygen tension (Lloyd and Butow 1974; Egner, Jakobs et al. 2002; David and Poyton 2005). The possibility that growth conditions may induce morphological plasticity of mitochondria-like organelles or preferential expression of one type over the other should be investigated by repeating EM and immunochemical experiments in cells at various stages in their lifecycles and grown under a range of O₂ tensions, and nutrients concentrations.

The second possibility for explaining the coexistence of mitosomes and hydrogenosomes in *S. vortens* is that a protomitochondrion may have appeared, as a product

of a first endosymbiotic event involving an aerobic endosymbiont. During the evolutionary adaptation to anaerobic lifestyles, such protomitochondria would have degenerated into mitosomes, retaining only the most basic Fe-S cluster assembly functions. Subsequently, a second endosymbiotic event involving an anaerobic bacterium could have occurred, thus leading to the formation of a hydrogenosome-like organelle.

The latter scenario would have interesting, wider implications for generalised theories of hydrogenosomal evolution. Indeed, the single-endosymbiotic event theory is the one currently supported by most (Martin and Muller 1998; Tielens, Rotte et al. 2002; Embley, van der Giezen et al. 2003a&b; van der Giezen, Cox et al. 2004). One of the arguments in favour of this theory is that the mutually exclusive distribution of mitochondria, mitosomes and hydrogenosomes indicates that these organelles represent different evolutionary steps in the same endosymbiotic event. Our discovery of the likely coexistence of mitosomes and hydrogenosomes in *S. vortens* may therefore lend more weight to the opposing theory, suggested by Dyall, Brown et al. 2004 and Dyall, Yan et al. 2004, which advances that mitochondria and mitosomes may have originated from two distinct endosymbiotic events. However, if our discovery favours the double-endosymbiotic event theory, several other strong arguments also exist in favour of the single endosymbiotic event theory. As, with the exception of *B. hominis* (Nasirudeen and Tan 2004; Wawrzyniak, Roussel et al. 2008), mitosome-like structures appear not to contain DNA, thus definite validation of either theory is unlikely to be obtained in the near future. Such evolutionary speculations therefore appear deemed to remain just that.

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CHAPTER 9: CONCLUDING COMMENTS

The current work provided novel information on the growth, metabolism, ultrastructure and chemotherapy of *Spironucleus vortens*, a poorly studied diplomonad flagellate, which infects the intestinal track of tropical fish and is responsible for hole-in-the-head disease in cichlids (Paull and Matthews 2001). The best studied, closest relative of *S. vortens*, is *Giardia intestinalis*, a diplomonad that also infects the intestinal track, albeit in humans, and has been used throughout the current study as a reference organism. Surprisingly, although these related parasites have similar ecological niches and morphologies, almost every aspect of the biology of *S. vortens* that was investigated in the current study was found to differ significantly from that of *Giardia intestinalis*.

A non-fastidious organism, *S. vortens* grew *in vitro* to high cell densities with an extremely low doubling time, but demonstrated an unusual biphasic pattern of growth, which may indicate the ability to switch its metabolism in order to adapt to a new nutrient source, thus suggesting a plastic metabolism. This plasticity was confirmed by mass spectrometric investigations, which showed that the organism could utilize very different types of substrates, such as glucose and ethanol, as energy sources. Surprisingly, NMR and bioscreen studies showed that neither of these compounds were the preferred substrates of this organism, and although a peptidic nature for its preferred fuel molecule was suggested by the results of NMR and amino acid analyses, the identity of such a substrate could not be established, possibly because the range of substrates tested was too restricted or the concentrations employed too high. Identification of the identity of such substrate will undoubtedly be achieved through further investigations, using a broader range of substrates, at lower concentrations. This may prove invaluable in understanding host-parasite interactions. Metabolic routes common in other anaerobic flagellates, such as the arginine dehydrolase pathway (Schofield, Costello et al. 1990; Schofield, Edwards et al. 1992; Yarlett, Lindmark et al. 1994; Yarlett, Martinez et al. 1996; Biagini, McIntyre et al. 1998; Brown, Upcroft et al. 1998; Biagini, Yarlett et al. 2003) seem absent in this organism, but *S. vortens* generates large amounts of hydrogen, which is highly unusual within the eukaryotic kingdom (Lloyd,

Hillman et al. 1989; Lloyd, Ralphs et al. 2002), and further highlights the atypical nature of the metabolism of this fascinating parasite.

Besides exhibiting a versatile metabolism, *S. vortens* also appeared to possess large pools of endogenous substrate, and unlike *G. intestinalis*, which relies exclusively on pinocytosis and other plasma membrane transport systems for nutrition (Gillin, Reiner et al. 1991; McCaffery and Gillin 1994; Becker and Melkonian 1996; Lanfredi-Rangel, Attias et al. 2003; Marti, Regos et al. 2003; Hernandez, Castillo et al. 2007), it proved capable of highly efficient phagocytosis. *S. vortens* is known to cause devastating systemic infections, and its metabolic plasticity, as well as the highly efficient phagocytosis probably contribute to the ability of the parasite to adapt to the large variety of ecological niches encountered in the course of systemic infections. Within nutrient-rich growth medium, *S. vortens* also demonstrated an extremely high proteolytic activity, presumably through the release of extracellular proteases. This may not solely be for nutrition, but could also constitute a virulence factor and thus contributes to the invasiveness of this organism (McKerrow, Bouvier et al. 1991; North 1991; North 1991). This invasiveness is also undoubtedly served by the remarkable motility of this organism, which was observed to burrow through solid agar, and was impossible to immobilize for long, even on chilled, methyl-cellulose coated slides, thus rendering live cell imaging highly challenging.

Due to the toxicity of metronidazole, no satisfactory chemotherapy is currently available for the treatment of this versatile parasite (Richardson and Bowron 1985; Dobias, Cerna et al. 1994; GESAMP 1997; Lanzky and Halling-Sorensen 1997; L82/14 1998; Cavas and Ergere Gozukara 2005; Huet, Mortier et al. 2005), and although it could be inhibited by the use of high concentrations of garlic and garlic-derived compounds, *S. vortens* demonstrated a relatively high degree of resistance for such products, which suggests that garlic-derived remedies are not a viable alternative for the treatment of spironucleosis. Because garlic contains a combination of several active compounds, whose mode of action may differ widely (Naganawa, Iwata et al. 1996; Ankri and Mirelman 1999; Avato, Tursil et al. 2000; O'Gara, Hill et al. 2000; Harris, Cottrell et al. 2001; Davis 2005; Hunter, Caira et al. 2005), the basis for such a high level of resistance is unlikely to be due to individual enzymes. It more probably derives from a form of physical protection, possibly awarded by atypical

properties of the membrane or cuticle. This suggests that the organism may also prove resistant to a range of inhibitory compounds, thus rendering the search for alternative treatments particularly difficult.

S. vortens was described as an aerotolerant anerobe (Poynton, Fraser et al. 1995), and the current study showed that it possessed highly efficient mechanisms for coping with large fluctuations in oxygen tensions. The nature of these mechanisms has not been investigated in this study but may be similar to what is observed in *T. vaginalis*, *G. intestinalis* and *H. inflata*, in which O₂ detoxification occurs largely through the action of NAD(P)H oxidase, presumably using NAD(P)H produced through glycolysis as electron donor. The presence of large pools of thiols subsequently insures that the OH[•] produced in that process is reduced to H₂O (Ellis, Wingfield et al. 1993; Ellis, Yarlett et al. 1994; Brown, Upcroft et al. 1995; Brown, Upcroft et al. 1996; Biagini, Suller et al. 1997; Tekwani and Mehlotra 1999; Muller, Liebau et al. 2003; Di Matteo, Scandurra et al. 2008). Interestingly, besides being tolerant of large concentrations of oxygen, *S. vortens* demonstrated an extremely high affinity for the gas, which suggests a metabolic function for the gas and indicates that this organism may be microaerophilic, rather than truly anaerobic.

Perhaps the most interesting finding of this study was that *S. vortens*, which was previously reported to lack any type of redox active organelle (Brugerolle, Joyon et al. 1973; Cavalier-Smith 1987; Poynton, Fraser et al. 1995), in fact appears to possess both hydrogenosomes and mitosomes. In the light of the discovery that *S. vortens* produces hydrogen at a very high rate, the discovery that it possesses hydrogenosomes, which are organelles specialised in hydrogen production, is hardly surprising, even though it was never previously reported. The coexistence of these hydrogen-generating organelles with mitosomes, is however far more interesting. Indeed, such a combination has so far never been described for any other eukaryote (van der Giezen and Tovar 2005), and may have important implications for the current hypotheses regarding the evolution of mitochondria-related organelles. It may indeed represent an intermediate evolutionary form between organisms that possess fully functional redox-organelles like *Trichomonas vaginalis*, and organisms in which, during the evolutionary adaptation to anaerobic lifestyles, these organelles have lost all their redox functions and degenerated into mitosomes, like *Giardia intestinalis*. The

evolutionary pressure responsible for the persistence of this intermediate state could be exposure to highly variable O₂ tensions, and the presence of both types of organelles may be key to understanding the remarkable tolerance of the organism to widely fluctuating O₂ tensions. Investigations of the presence of these organelles in other species of *Spironucleus* species; such as *Spironucleus torosa*, which within the intestine of its marine host may be exposed to more constant O₂ tensions, may prove extremely valuable in testing this hypothesis.

The current study has uncovered many atypical traits in *S. vortens*, several of which raise fascinating questions, and call for more detailed examination of its metabolism, ultrastructure and interaction with its host. Further work should include in depth characterisation of *S. vortens* fermentative metabolism, as well as of the influence of oxygen, not just on metabolism, but also on growth. Virulence and invasiveness should also be investigated in a range of hosts, and efficiency of alternative chemotherapeutic agents, such as garlic should be tested *in vivo*.

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PUBLICATIONS

RESEARCH PAPERS:

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ABSTRACTS AND ORAL PRESENTATIONS:

American society of Protistology, Lisbon 2006: Hydrogen production in the fish parasite *Spironucleus vortens*

British society of Parasitology, Belfast 2007: Metabolism and ultrastructure of the fish parasite *Spironucleus vortens*

European congress of Protistology and IX European conference on ciliate biology, St Petersburg, 2008: Biology and ultrastructure of the fish parasite *Spironucleus vortens*

ABSTRACTS AND POSTER PRESENTATIONS:

Photochemistry and Photophysics of coordination compounds, Dublin 2007: Quantum-dots assays to investigate uptake mechanisms in *Spironucleus vortens* and *Giardia intestinalis*

APPENDICES

APPENDIX 1. PRELIMINARY DATA ON RELATIVE PREVALENCE OF *S. BARKHANUS* IN BRITISH AND NORWEGIAN GRAYLING AND REPORT ON *IN VITRO* CULTIVATION ATTEMPTS OF *S. TOROSA*

BACKGROUND

Spironucleus species have previously been isolated from wild and farmed marine, and freshwater fish in Norway and Canada (Mo, Poppe et al. 1990; Kent, Ellis et al. 1992; Poppe, Mo et al. 1992; Sterud and Poppe 1997; Sterud 1998a&b; Sterud, Mo et al. 1998; Sterud and Poynton 2002; Sterud, Poppe et al. 2003; Jorgensen and Sterud 2006; Fard, Jorgensen et al. 2007), but the prevalence of infection in UK fish has never been investigated. In the current study, *S. barkhanus* infection status of wild and hatchery-born grayling was investigated in the UK and Norway. Isolation of parasites in pure cultures was attempted for *S. barkhanus* and *S. torosa*; the latter species infects marine gadids (Poynton and Morrison 1990; Sterud 1998a).

MATERIAL AND METHODS

Screening of British and Norwegian grayling for *Spironucleus barkhanus*

The gall bladder of three wild grayling (*Thymallus thymallus*, Oslo, Norway), two Norwegian grayling captured near Oslo and maintained in an aquarium (Natural History Museum, University of Oslo) for three years, 3 wild Welsh grayling from River Taff, Cardiff, 21 wild Welsh grayling from a small feeder stream of the Taff, Bute Park, Cardiff, as well as 20 hatchery-born grayling (Nottingham, UK), were dissected and examined for the presence of *S. barkhanus*. Fish were killed by a sharp blow to the head and gall bladders were sampled aseptically. Using a sterile syringe the contents of each gall bladder (0.1 to 0.5 ml) were transferred to 10 ml TY-I-S33 culture medium, and incubated at 5°C or 10°C for 144 h. A

small amount of gall bladder fluid was retained for light microscopy examination. Cultures were checked daily by light microscopy for the presence of live trophozoites.

Isolation of *Spironucleus torosa* from marine fish

The intestine of five atlantic cods (*Gadus morhua*), two saithes (*Pollachius virens*), five mackerels (*Scomber scombrus*) and one plaice (*Pleuronectes platessa*) captured in the bay of Oslo were dissected and examined for the presence of *S. torosa*. When microscopic examination of intestinal content revealed the presence of *S. torosa* trophozoites, approximately 0.5 ml of intestinal fluid was removed, introduced into 10 ml of TY-I-S33 culture medium, and incubated at 5°C for 12 h with 1000 U penicillin and 150 µg. ml⁻¹ gentamicin. The cultures were inoculated at the top end of a sterile glass S-shaped tube (as described in Chapter 2), and trophozoites were collected at the distal end after 12 h, before being inoculated into fresh TY-I-S33, with or without the addition of 5% (w/w) autoclaved mashed liver and intestinal content from the host fish.

Experimental infections of guppies with *Spironucleus vortens*

Four seemingly healthy female guppies (*Poecilia reticulata*), approximately 3.5 cm standard length, were randomly selected from a tank and placed in individual tanks at 20°C for 48 h. Fish were then anaesthetized with 0.02% tricaine methanosulfonate (MS222), buffered to a neutral pH with NaHCO₂, scratched lightly on the tail with a sharp needle, and a suspension of *S. vortens* trophozoites was swabbed on the wounds of 3 of the fish, while the fourth fish was sham infected without exposure to the parasite.

RESULTS

2. 4 |Screening of British and Norwegian grayling for *Spironucleus barkhanus*

None of the British grayling, wild or hatchery-born, appeared to carry *S. barkhanus*. Live trophozoites were not seen in the bile and inoculation of bile in TY-I-S33 culture medium, followed by incubation at 5 or 10°C for 144 h failed to yield any live trophozoites. The aquarium-kept Norwegian grayling were also free from *S. barkhanus* infection, as were two of

the wild grayling. However, in a third wild Norwegian grayling, a large number of actively swimming trophozoites were detected in the gall bladder, and parasites were successfully maintained in culture for several months at 5 and 10°C.

2. 5 Isolation of *Spironucleus torosa* from marine fish.

All five cods and three saithes were found to harbour large numbers of *S. torosa* in their intestine. Parasites were successfully recovered from Keister's modified culture medium and from pure cultures after antibiotic treatment and separation through S-tube. However, culturing appeared to affect parasite morphology and behaviour. Parasites directly collected from the fish were narrow and swam very rapidly, whereas those that had been *in vitro* cultures for 48 h appeared swollen and swam more slowly. These changes were not altered by the addition of autoclaved gut content and mashed liver from the host fish. All cultures failed after 6 days. No trophozoites could be found after thorough microscopic examination of the intestine content of haddocks and plaice.

2. 6 Experimental infections of guppies with *Spironucleus vortens*

One fish exposed to the parasite died two days after infection, however it presented none of the symptoms of Spironucleosis, and no parasites were detected by light microscopy in samples of skin, blood or gut content. Therefore, this guppy probably died from natural causes or due to the stress occasioned by the anaesthesia or the scratching procedure. In all remaining fish, wounds healed within 4 days, and fish remained healthy and continued feeding.

CONCLUSIONS

Although *Spironucleus barkhanus* infections appear common amongst Norwegian wild grayling, no infected fish were detected in samples of 24 wild and 20 hatchery-born UK grayling, which suggests that the prevalence of infection is much lower in the UK. Studies using larger samples and genetic markers for accurate identification of parasites will be required to further investigate the distribution of this parasite.

Successful *in vitro* culturing of *S. torosa* requires further optimization in growth conditions, and based on the swollen appearance of trophozoites when in culture medium; it is possible that osmolarity of culture medium may have to be adjusted for growth of this marine organism (although in marine fish, most of the salt in swallowed sea water is pumped out in the stomach).

Wound swabbing with trophozoites of the angelfish strain of *S. vortens* did not result in the establishment of infections in guppies, however, potential cross-infectivity of *S. vortens* in guppies and other fish should be further investigated, using other portal of entries, and parasites isolated from infected fish, rather than trophozoites from an ATCC culture, which has been maintained *in vitro* for the past 19 years, and may thus have lost part of their virulence or infectivity. For instance, infectivity of cysts in food pellets could be tested. Indeed, in other diplomonads, such as *Giardia intestinalis*, the trophozoites are not infectious, and disease can only be transmitted through ingestion of cysts.

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APPENDIX 2: AMPLIFICATION OF *SPIRONUCLEUS VORTENS* DNA USING SSU 18S rDNA PRIMERS DEVELOPPED IN *SPIRONUCLEUS BARKHANUS*

INTRODUCTION

Disease management in the fish industry is extremely problematic, as diagnosis can rely on very few external clinical signs and the cause of disease is difficult to establish. In most cases, a disease is only detected when abnormal feeding behaviour or impaired growth is observed. Diagnosis and treatment needs to be as rapid as possible, as disease in fish farms or ornamental aquaria almost always results in a disease outbreak, due to the crowded host conditions (GESAMP 1997; Treves-Brown 1999). Hexamitids are common intestinal track commensals of fish, and only a few species are known to behave as opportunistic pathogens (Poynton and Sterud 2002). Isolation of hexamitid flagellates is thus not sufficient to establish the cause of an outbreak and precise identification of the species is necessary. Identification of hexamitid flagellates has always been challenging, which has led to much confusion in their taxonomy and host range, as well as impaired diagnosis and disease management (Poynton and Sterud 2002; Poynton, Fard et al. 2004). Much of the earliest taxonomical work was performed using light microscopy (Moore 1922; Davis 1926; Vickerman 1990), a method which is unreliable for the identification of these small parasites and has also resulted in poor species definitions, which has escalated the taxonomic problems (Poynton and Sterud 2002). For instance, there is currently strong evidence that most *Hexamita* isolates from fish are actually *Spironucleus* spp. (Poynton and Morrison 1990; Sterud 1998; Sterud 1998; Sterud, Mo et al. 1998), Poynton and Sterud 2002). Scanning and transmission electron microscopy was, until recently, the only accurate means of identifying these parasites, however this is a time consuming and labour intensive process that requires specialist knowledge and is often incompatible with the need for a quick and reliable diagnosis necessary in disease management.

DNA sequencing provides an alternative, reliable mean of identifying hexamitids, but the technique is not yet widely developed in veterinary diagnostic units. The value of such methods is highlighted by the study by (Jorgensen and Sterud 2004), in which the SSU RNA

sequence of *S. barkhanus* isolates from an outbreak in caged Arctic char was compared to that of isolates from surrounding wild char populations. The study revealed that isolates from diseased caged fish were genetically distinct from those of wild char population surrounding the cages, but 99.7% similar to the strain responsible for outbreaks in Atlantic and Chinook salmon (Norway and Canada) (Jorgensen and Sterud 2004). These results therefore suggested the existence of epidemic strains. However, no molecular diagnosis tool is available commercially and existing primers based on SSU 18S r DNA are species specific at best. Systematic molecular surveys of *Spironucleus* parasites have recently been published by (Jorgensen and Sterud 2007), but to date, the development of species-specific primers has not been undertaken. In an effort to assess specificity of existing primers, and with a view to develop primers specific to *S. vortens*, we attempted amplification of DNA from *S. vortens* and from the related free-living diplomonad *Hexamita inflata* using primers developed in *S. barkhanus*.

MATERIALS AND METHODS

DNA was isolated from 10^6 cells using a Qiagen DNAeasy Tissue Extraction Kit and eluted into 200 μ l of AE buffer provided with the kit. For 18S rDNA amplification, primers Spironucleosis-1f (5' TCATTATCAGTGGTTAGTACATGC 3'; 5' end of 18S) and Spironucleosis-2r (5' TTCAAGCCTAACCACGACAAG 3'; 3' end of 18S), a generous gift from Dr Erik Sterud and Dr Anders Jorgensen (National Veterinary Institute, Oslo, Norway), were used in 10 μ l PCRs. These contained ca. 10 ng DNA, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.3 mM of each primer and 1U *Taq* DNA polymerase (Invitrogen). The standard thermal profiles and cycle times used were 94°C for 3 min followed by 94°C for 30 s, 50°C for 1 min, 72°C for 2 min (35 cycles), and 72°C for 10 min in a Robocycler thermal cycler. PCR success was determined by running products on an agarose gel. Fragment length of PCR product was determined by interpolation using a 100 bp ladder.

RESULTS & FUTURE WORK.

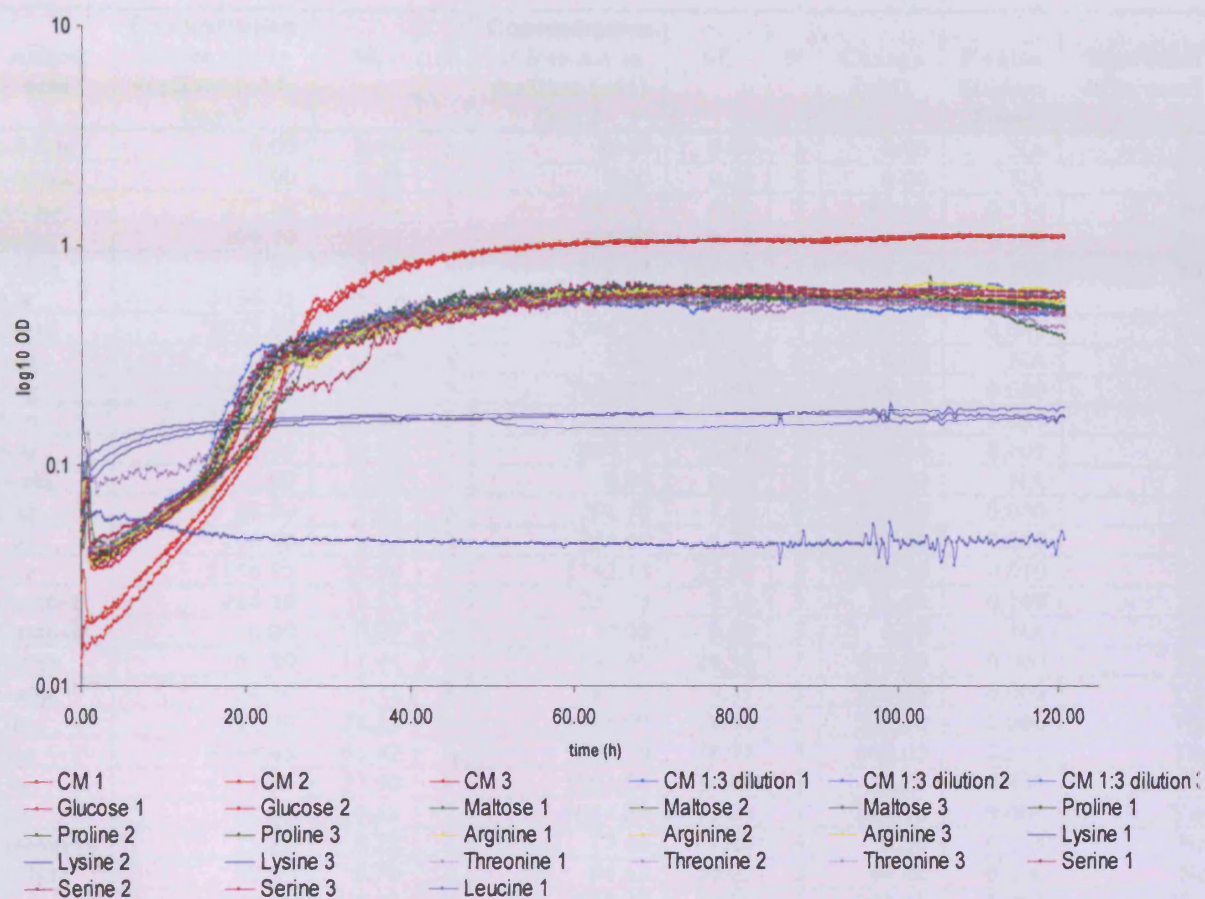
We successfully amplified DNA fragments of 1200kb and 1100kb fragments, respectively in *S. vortens* and *H. inflata*, and obtained clear negatives in control lanes. The primers used were designed for the sequencing of SSU rDNA of *S. barkhanus*, thus confirming the lack of specificity in these primers. Design of primers specific to established pathogenic strains would be invaluable in diagnosis, identification of potential reservoir hosts and disease management. Future work includes the design of such primers for pathogenic strains of *S. vortens*, the causative agent of hole-in-the-head disease in cichlids and *S. barkhanus*, which is responsible for devastating outbreaks of systemic infections in salmon farms. Construction of these specific primers should be attempted using the more variable internal transcribed spacers of the SSU rDNA.

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**APPENDIX 5. 4. 4 OUTPUT OF AUTOMATED OPTICAL DENSITY MONITORING
OF GROWTH OF *SPIRONUCLEUS VORTENS* IN DILUTED CULTURE MEDIUM
COMPLEMENTED WITH VARIOUS SUBSTRATES**



CM= culture medium

**APPENDIX 5. 4. 5 CHANGES IN FREE AMINO ACID CONCENTRATIONS IN
CONDITIONNED KEISTER'S MODIFIED TY-I-S33 CULTURE MEDIUM**

5. 4. 5. A. AFTER 4 DAYS OF GROWTH OF *SPIRONUCLEUS VORTENS*

Amino acid	Concentration of free AA in medium (μM) Day 0	SE	N	Concentration of free AA in medium (μM) Day 4	SE	N	Change (μM)	P value Student T-test	Significant difference?
1-Mhis	0.00	0.00	3	0.00	0.00	3	0.00	NA	No
3-Mhis	0.00	0.00	3	0.00	0.00	3	0.00	NA	No
AAAA	277.60	39.52	3	217.50	6.44	3	-60.10	0.374	No
Aaba	206.50	12.10	3	176.00	0.76	3	-30.50	0.169	No
Aaiba	0.00	0.00	3	382.65	127.55	3	382.65	0.116	No
Ala	5136.55	65.59	3	7401.30	85.04	3	2264.75	0.000	Yes
Amm	3923.45	57.38	3	4948.20	90.13	3	1024.75	0.003	Yes
Ans	0.00	0.00	3	0.00	0.00	3	0.00	NA	No
Arg	2944.10	27.14	3	2045.70	1.85	3	-898.40	0.000	Yes
Asn	1395.70	22.84	3	1262.75	7.18	3	-132.95	0.021	Yes
Asp	1548.45	18.58	3	1867.85	16.85	3	319.40	0.001	Yes
B-ala	0.00	0.00	3	0.00	0.00	3	0.00	NA	No
Car	88.80	3.64	3	201.30	4.42	3	112.50	0.000	Yes
Citr	115.70	3.72	3	329.60	6.70	3	213.90	0.000	Yes
Cys	3156.85	54.90	3	2742.15	23.81	3	-414.70	0.010	Yes
Cysth-1	214.10	2.91	3	235.70	7.53	3	21.60	0.149	No
Cysth-2	0.00	0.00	3	0.00	0.00	3	0.00	NA	No
Ethan	162.80	11.44	3	638.60	24.36	3	475.80	0.000	Yes
Gaba	54.30	1.12	3	280.75	9.11	3	226.45	0.000	Yes
Gln	511.70	28.25	3	1015.00	14.40	3	503.30	0.000	Yes
Glu	5198.45	61.42	3	5698.50	48.97	3	500.05	0.013	Yes
Gly	1911.75	27.90	3	1901.00	14.48	3	-10.75	0.831	No
His	792.80	10.55	3	1014.20	11.84	3	221.40	0.001	Yes
Homocys	11.25	0.32	3	17.45	1.48	3	6.20	0.053	No
Hylys	50.55	0.70	3	94.65	20.02	3	44.10	0.216	No
Ile	2540.00	26.83	3	2930.45	30.36	3	390.45	0.003	Yes
Leu	8707.65	84.05	3	7995.20	69.50	3	-712.45	0.012	Yes
Lys	5076.20	54.35	3	4481.15	35.41	3	-595.05	0.004	Yes
Met	1473.95	18.66	3	1771.50	11.14	3	297.55	0.001	Yes
NLeu	16.95	0.00	3	16.95	0.00	3	0.00	NA	No
OHPro	0.00	0.00	3	0.00	0.00	3	0.00	NA	No
Orn	257.00	4.38	3	217.95	5.85	3	-39.05	0.024	Yes
Pea	540.05	57.59	3	462.65	0.91	3	-77.40	0.421	No
Phe	3503.55	32.68	3	3254.95	45.38	3	-248.60	0.041	Yes
PhSer	0.00	0.00	3	0.00	0.00	3	0.00	NA	No
Pro	2884.65	277.72	3	4011.15	79.10	3	1126.50	0.060	No
Sarc	1434.95	364.47	3	1592.45	10.01	3	157.50	0.788	No
Ser	2392.90	30.45	3	2042.90	18.91	3	-350.00	0.003	Yes
Tau	387.75	1.40	3	104.20	4.33	3	-283.55	0.000	Yes
Thr	2495.65	29.79	3	2191.65	18.15	3	-304.00	0.004	Yes
Trp	1045.15	18.66	3	1041.50	12.02	3	-3.65	0.918	No
Tyr	864.30	9.87	3	883.25	15.72	3	18.95	0.533	No
Urea	2325.30	21.78	3	1731.45	26.10	3	-593.85	0.000	Yes
Val	3732.95	42.31	3	3734.40	32.82	3	1.45	0.986	No
TOTAL	67380.35			70934.6			3554.25		

5. 4. 5. B. AFTER 6 DAYS OF GROWTH OF *SPIRONUCLEUS VORTENS*

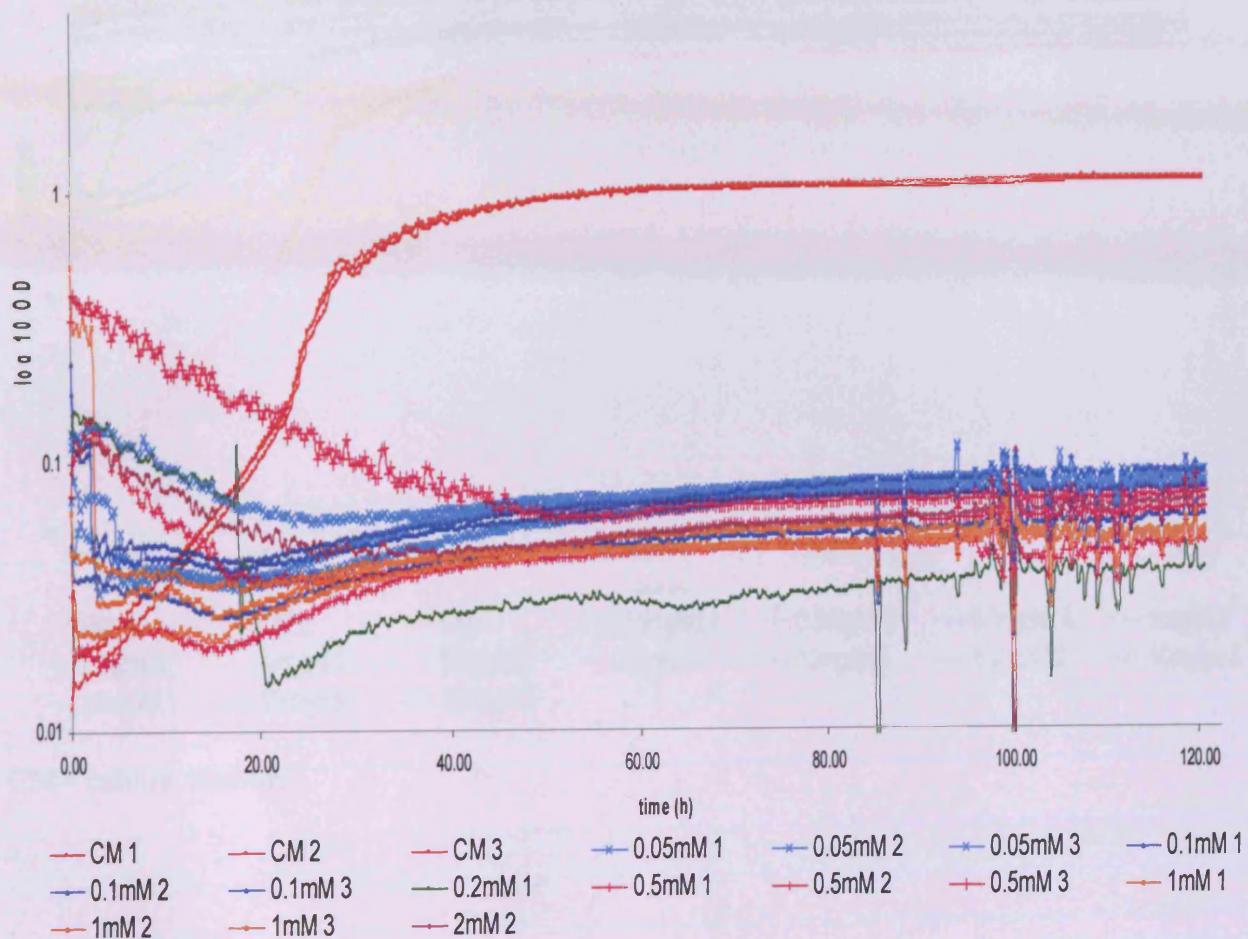
Amino acid	Concentration of free AA in medium (μM) Day 0	Concentration of free AA in medium (μM) Day 6	SE	N	Change (μM)	P value Student T-test	Significant difference?
1-Mhis	0.00	0.00	0.00	3	0.00	NA	No
3-Mhis	0.00	0.00	0.00	3	0.00	NA	No
AAAA	133.80	204.53	23.47	3	70.73	0.095	No
Aaba	0.00	125.10	4.50	3	125.10	0.001	Yes
Aaiba	0.00	460.63	26.17	3	460.63	0.003	Yes
Ala	4687.80	8648.60	348.48	3	3960.80	0.008	Yes
Amm	4556.70	6940.80	291.02	3	2384.10	0.015	Yes
Ans	0.00	0.00	0.00	3	0.00	NA	No
Arg	3318.20	2340.73	47.43	3	-977.47	0.002	Yes
Asn	1173.90	1450.60	71.34	3	276.70	0.061	No
Asp	1388.30	1918.57	43.44	3	530.27	0.007	Yes
B-ala	345.10	368.43	184.99	3	23.33	0.911	No
Car	101.20	166.20	5.50	3	65.00	0.007	Yes
Citr	0.00	255.37	3.70	3	255.37	0.000	Yes
Cys	3083.90	2467.87	47.11	3	-616.03	0.006	Yes
Cysth-1	0.00	69.63	69.63	3	69.63	0.423	No
Cysth-2	213.00	80.30	40.15	3	-132.70	0.081	No
Ethan	0.00	738.43	128.44	3	738.43	0.029	Yes
Gaba	51.00	228.57	37.06	3	177.57	0.041	Yes
Gln	282.80	939.17	94.98	3	656.37	0.020	Yes
Glu	5214.00	6532.40	156.76	3	1318.40	0.014	Yes
Gly	1912.70	2437.73	49.81	3	525.03	0.009	Yes
His	816.80	1220.37	44.63	3	403.57	0.012	Yes
Homocys	12.20	18.97	2.38	3	6.77	0.105	No
Hyllys	118.40	104.73	29.90	3	-13.67	0.102	No
Ile	2564.60	3256.10	44.17	3	691.50	0.004	Yes
Leu	8991.80	9502.17	189.35	3	510.37	0.114	No
Lys	5077.70	494.60	22.81	3	-4583.10	0.000	Yes
Met	1564.30	1752.27	18.44	3	187.97	0.009	Yes
NLeu	18.80	18.80	0.00	3	0.00	NA	No
OHPro	0.00	0.00	0.00	3	0.00	NA	No
Orn	159.00	267.37	22.92	3	108.37	0.052	No
Pea	342.70	229.93	74.68	3	-112.77	0.270	No
Phe	3701.10	3715.60	97.72	3	14.50	0.870	No
PhSer	0.00	0.00	0.00	3	0.00	NA	No
Pro	1089.70	2122.03	146.22	3	1032.33	0.019	Yes
Sarc	381.60	1556.00	333.10	3	1174.40	0.072	No
Ser	2258.10	2427.40	56.89	3	169.30	0.097	No
Tau	192.10	120.90	13.00	3	-71.20	0.032	Yes
Thr	2351.10	2507.90	53.98	3	156.80	0.101	No
Trp	1217.30	1339.20	30.17	3	121.90	0.056	No
Tyr	901.60	987.80	15.79	3	86.20	0.032	Yes
Urea	1728.10	2344.57	301.13	3	616.47	0.177	No
Val	3696.40	4428.87	102.33	3	732.47	0.019	Yes
TOTAL	63645.8	74789.24			11143.44		

**APPENDIX 5. 4. 6 CHANGES IN TOTAL AMINO ACID CONCENTRATION IN
HYDROLYSATE OF KEISTER'S MODIFIED CULTURE MEDIUM AFTER 4 DAYS
OF GROWTH**

Amino acid	Concentration of free AA in medium (μM) Day 0	Concentration of free AA in medium (μM) Day 6	Change (μM)	AA lost in hydrolysis
1-Mhis	0.00	0.00	0.00	
3-Mhis	0.00	0.00	0.00	
AAAA	75.24	0.00	-75.24	
Aaba	60.06	113.85	53.79	
Aaiba	175.89	0.00	-175.89	
Ala	18252.96	21352.32	3099.36	
Amm	33880.11	31886.58	-1993.53	
Ans	0.00	0.00	0.00	
Arg	8319.63	6547.20	-1772.43	
Asn	0.00	48.51	48.51	
Asp	2209.68	19668.00	17458.32	
B-ala	0.00	325.71	325.71	
Car	0.00	0.00	0.00	
Citr	455.73	285.78	-169.95	
Cys	7056.06	6106.98	-949.08	
Cysth-1	112.86	99.99	-12.87	
Cysth-2	19.47	24.75	5.28	
Ethan	177.21	161.70	-15.51	
Gaba	220.77	211.86	-8.91	
Gln	0.00	0.00	0.00	
Glu	48940.32	45528.45	-3411.87	
Gly	13367.97	12203.07	-1164.90	
His	6123.81	5562.81	-561.00	
Homocys	0.00	0.00	0.00	
Hyls	0.00	19.80	19.80	
Ile	14130.93	12994.41	-1136.52	
Leu	26182.53	24055.68	-2126.85	
Lys	19098.75	17631.24	-1467.51	
Met	3709.53	3177.90	-531.63	
NLeu	37.29	37.29	0.00	
OHPro	0.00	0.00	0.00	
Orn	474.87	470.25	-4.62	
Pea	458.04	326.37	-131.67	
Phe	10449.12	9338.34	-1110.78	
PhSer	0.00	0.00	0.00	
Pro	23833.59	21687.60	-2145.99	
Sarc	420.75	391.38	-29.37	
Ser	17833.86	15122.91	-2710.95	
Tau	100.98	92.40	-8.58	
Thr	16254.48	14218.38	-2036.10	
Trp	0.00	104.94	104.94	
Tyr	4135.89	3693.03	-442.86	
Urea	0.00	0.00	0.00	
Val	20534.58	18845.64	-1688.94	
TOTAL	297103	292335.1	-4767.84	

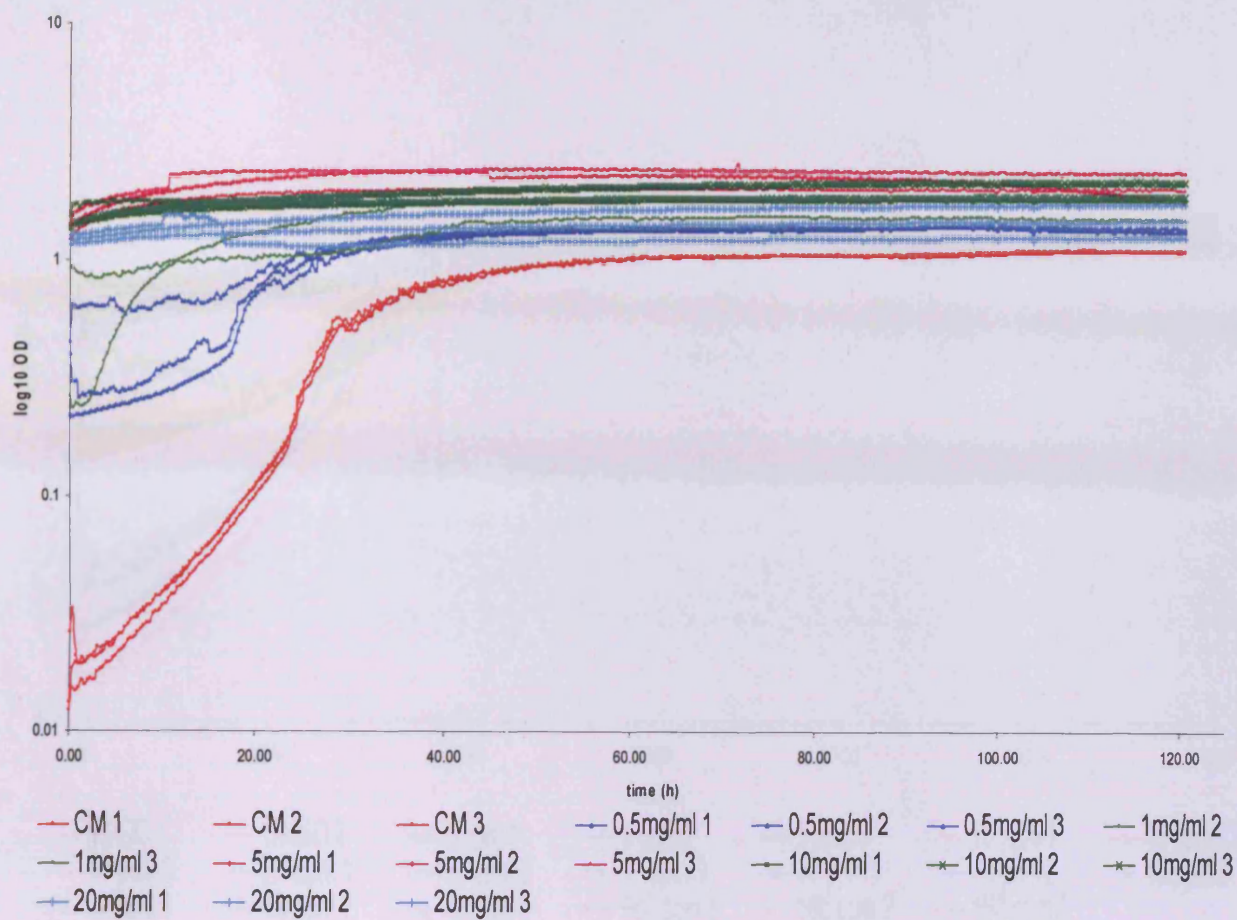
APPENDIX 7. OUTPUT OF AUTOMATED OPTICAL DENSITY MONITORING OF GROWTH OF *SPIRONUCLEUS VORTENS* UPON ADDITION OF INHIBITORS

7. A. METRONIDAZOLE



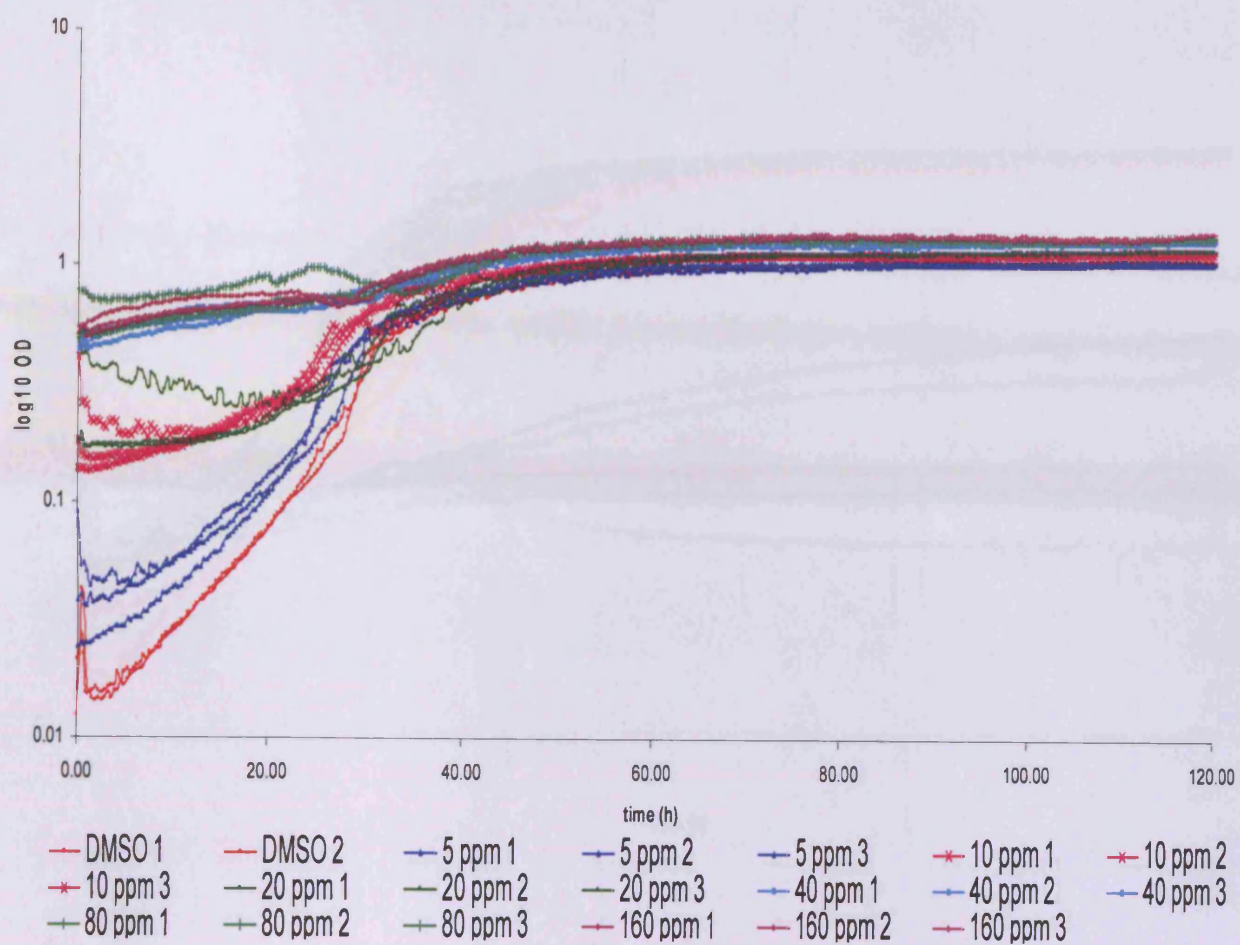
CM= culture medium

7. B. FREEZED-DRIED GARLIC



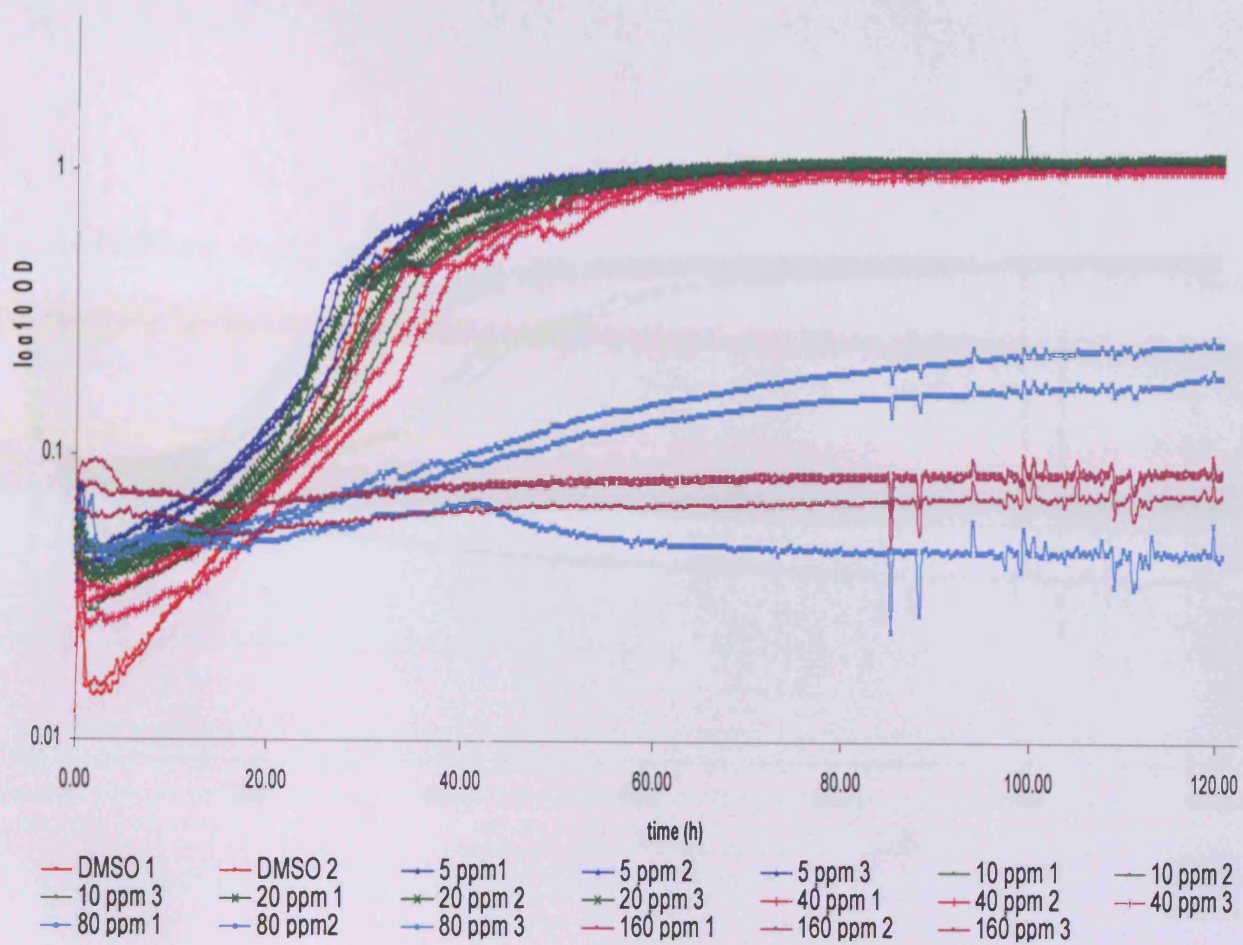
CM= culture medium

7. C. ALLICIN



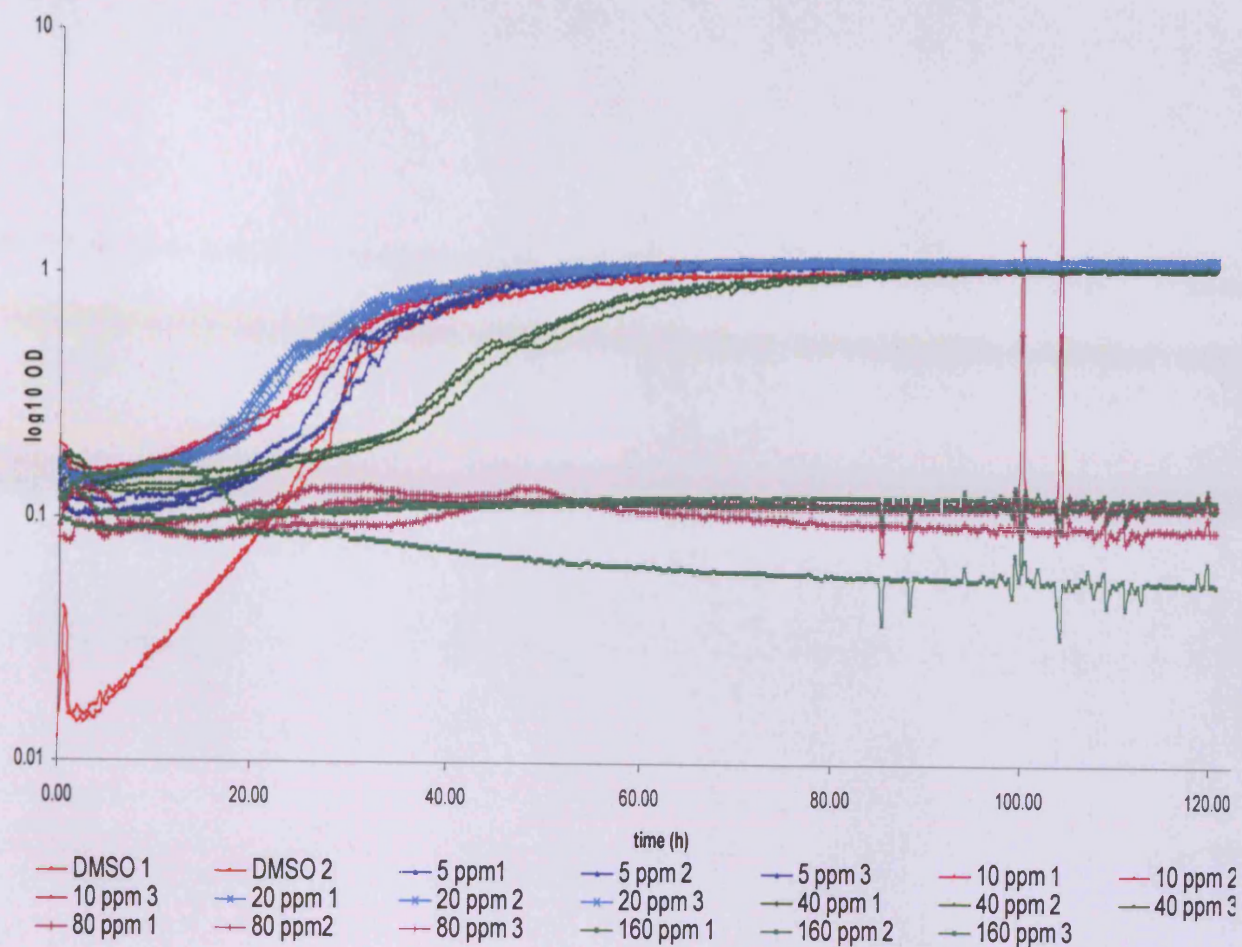
CM= culture medium

7. D. AJOENE



CM= culture medium

7. E. AJOENE-FREE MIXTURE OF THIOSULFINATES AND DITHIINS



CM= culture medium

