

# **Ciliate-zooplankton epibiosis in Lake St Lucia**

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## ABSTRACT

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Epibiosis is a symbiotic association of two organisms in which one species (epibiont) uses the surface of another species (basibiont or host) as an attachment substrate. An increasing number of studies are revealing that epibionts have mainly deleterious effects on crustacean mesozooplankton (hereafter referred to simply as zooplankton) hosts. In spite of its widespread occurrence, there are very few studies in Africa that address epibiosis in the aquatic environment, particularly involving zooplankton as hosts. Epibiotic ciliates are often found attached to zooplankton in the St Lucia Estuary, in northern KwaZulu-Natal, South Africa. St Lucia is the largest estuarine lake in Africa and is globally recognized for its ecological importance. A study was conducted in St Lucia between 2015 and 2017, with the aim of determining: the identity of the epibiotic ciliates; their species-specific association with the zooplankton of St Lucia; the effects they have on their hosts and the environmental conditions that promote their proliferation. Based on live observations and images obtained from protargol staining and scanning electron microscopy, the epibiotic ciliates in the St Lucia Estuary were identified as the peritrich sessilid *Epistylis* sp. (Chapter 1). The results of the experimental study in Chapter 2 were that *Epistylis* sp. is species-specific, attaching only to the dominant calanoid copepod *Pseudodiaptomus stuhlmanni* (mainly adults) and that this relationship is host density dependent. Another finding of Chapter 2 was that *Epistylis* sp. exerts a negative effect on the survivorship of heavily covered *P. stuhlmanni*. The results of Chapter 3 revealed a low RNA content and RNA:DNA ratio in epibiont-hosting *P. stuhlmanni* compared with their non-hosting counterparts, which implies a compromised nutritional status of epibiont-hosting copepods. Laboratory-based experiments detailed in Chapters 4 and 5 revealed that *Epistylis* sp. is: a) unaffected by temperature; and b) favoured by salinities below 20 and organically rich turbidity within the range 250–500 NTU. Results obtained from monthly field observations throughout 2016 (Chapter 6) showed no correlation of *Epistylis* sp. with these physico-chemical parameters and with the abundance of *P. stuhlmanni*. The latter result may be due to the uncharacteristically low abundance of the host *P. stuhlmanni* during the sampling period (January–December 2016). Overall, findings of this study suggest that peritrich epibionts can substantially and negatively affect host species and that they have a complex, context-dependent relationship with environmental conditions. The ecological implications of ciliate-zooplankton epibiosis in the St Lucia Estuary and in similar systems are discussed.

## SUMMARY OF THE MAIN FINDINGS:

- A) The peritrich epibiont attaching to zooplankton in the St Lucia Estuary belongs to the genus *Epistylis* (Chapter 1).
- B) *Epistylis* sp. attaches specifically to the calanoid copepod *Pseudodiaptomus stuhlmanni* despite availability of other zooplankton species. *Epistylis* sp. also prefers adult forms to copepodites (Chapters 2 and 6).
- C) *Epistylis* sp. compromises the fitness of *P. stuhlmanni* and this was detected at different levels of biological organisation. High cover ( $> 40\%$  body cover) by *Epistylis* sp. reduces the longevity of *P. stuhlmanni* (Chapter 2), and low–moderate ( $< 20 - < 40\%$ ) cover reduces the nutritional status of this copepod (Chapter 3).
- D) In controlled experiments, turbidity with a low nutritional value (inorganic silt) and high turbidity ( $\geq 1000$  NTU; inorganic and natural silt) has a negative effect on the attachment success of *Epistylis* sp. Intermediate (250–500 NTU) nutritious (natural silt) turbidity has a positive impact on attachment success (Chapter 4).
- E) In controlled experiments, turbidity and salinity but not temperature affect *Epistylis* sp. survivorship. High turbidity and salinity ( $\geq 20$ ) increase the mortality rate of this epibiont. *Epistylis* sp. has the highest survivorship at intermediate nutritious turbidities and displays no salinity preference within the range 0–15 (Chapter 5).
- F) An uncharacteristically low abundance of *P. stuhlmanni* was observed in the southern stations, Dredger Harbour and Sugarloaf Jetty throughout 2016. Low *Epistylis* sp. prevalence and cover were found and there was no correlation between this epibiont and all tested biotic (including *P. stuhlmanni* abundance) and abiotic factors (Chapter 6).

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## PREFACE

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The experimental work described in this PhD thesis was carried out at the University of KwaZulu-Natal: School of Life Sciences (Westville Campus) from May 2015 to November 2017, under the supervision of Dr Andre Vosloo, Dr Nicola Carrasco and Prof. Renzo Perissinotto.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, this is duly acknowledged in the text.

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## DECLARATION 1: PLAGIARISM

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I, **Salome Jones** declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research;
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## DECLARATION 2: PUBLICATIONS

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis are as follows:

### **Publication 1**

Jones S, Utz L, Carrasco NK, Perissinotto R, Vosloo A (In preparation) A first record and initial morphological characterisation of the epibiotic peritrich *Epistylis* sp. (Ciliophora:Peritrichia) in the St Lucia Estuary, South Africa.

Author contributions: Concept and design: SJ and LU; Laboratory and microscopy work: SJ; Draft paper: SJ; Editorial input: NKC, RP and AV.

### **Publication 2**

Jones S, Carrasco NK, Perissinotto R, Vosloo A (2016) Association of the epibiont *Epistylis* sp. with a calanoid copepod in the St Lucia Estuary, South Africa. *Journal of Plankton Research*, 38(6): 1404–1411.

Author contributions: Concept and design: SJ; Experimental work and data analyses: SJ; Draft paper: SJ; Editorial input: NKC, RP and AV.

### **Publication 3**

Jones S, Vosloo A, Carrasco NK, Perissinotto R (In preparation) Effects of the epibiont *Epistylis* sp. on the calanoid host *Pseudodiaptomus stuhlmanni* assessed using the RNA:DNA ratio and RNA content in the St Lucia Estuary, South Africa.

Author contributions: Concept and design: AV and SJ; Laboratory work and data analyses: SJ; Draft paper: SJ; Editorial input: AV, NKC and RP.

### **Publication 4**

Jones S, Carrasco NK, Vosloo A, Perissinotto R (2018) Impacts of turbidity on an epibiotic ciliate in the St Lucia Estuary, South Africa. *Hydrobiologia*. DOI: 10.1007/s10750-018-3545-8.

Author contributions: Concept and design: SJ; Experimental work and data analyses: SJ; Draft paper: SJ; Editorial input: NKC, AV and RP.

### **Publication 5**

Jones S, Carrasco NK, Vosloo A, Perissinotto R (In preparation) Effects of salinity, temperature and turbidity on the survivorship of the epibiotic peritrich *Epistylis* sp. in the St Lucia Estuary, South Africa.

Author contributions: Concept and design: SJ; Experimental work and data analyses: SJ; Draft paper: SJ; Editorial input: NKC, RP and AV.

### **Publication 6**

Jones S, Carrasco NK, Perissinotto R, Vosloo A (In preparation) Dynamics of the peritrich epibiont *Epistylis* sp. and its host, the calanoid *Pseudodiaptomus stuhlmanni* in the St Lucia Estuary, South Africa.

Author contributions: Concept and design: SJ; field work, microscopy and data analyses: SJ; Draft paper: SJ; Editorial input: NKC, RP and AV.

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## General Introduction

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### **Epibiosis: an overview**

Community ecology is the study of interspecific interactions such as predation, competition and symbiosis (Morin 2009). Epibiosis is an ecto-symbiotic association of different species whereby one, the epibiont spends at least one ontogenetic phase attached to a living substratum, the basibiont or host (Threlkeld et al. 1993). Epibiosis involving the attachment of organisms such as rotifers, protozoans and bacteria to zooplankton hosts is widespread in aquatic environments (Henebry and Ridgeway 1979; Green and Shiel 2000; Fernandez-Leborans et al. 2005; Bielecka and Boehnke 2014).

There are many benefits of assuming a sessile life while attached to a living, motile surface. In terms of energetics, host motility provides expense-free gene flow as well as transportation to high-nutrient zones (Barea-Arco et al. 2001; Regali-Selegim and Godinho 2004; Fernandez-Leborans 2010; Bickel et al. 2012). Zooplankton hosts may directly benefit their epibionts by providing nutrition through their feeding currents and the biofilms on their surface, as well as by protecting the epibionts from predation (Barea-Arco et al. 2001; Fernandez-Leborans 2010).

The host organism may also benefit from the epibiotic interaction. By altering the physical appearance of their crustacean hosts, epibionts camouflage their hosts from visual predators (Stoecker 1978). The chemical properties of the epibiont may also provide protection from predators who rely on chemical cues released by their prey (Wahl 1989 and references therein). In spite of the potential benefits to the host, epibiosis largely benefits the epibiont and many studies have demonstrated that epibionts commonly exert deleterious effects on their zooplankton hosts (Xu and Burns 1991; Allen et al. 1993; Threlkeld and Willey 1993; Visse 2007).

Rather than protect their hosts from predation, epibionts may compromise the motility and therefore the ability of their hosts to escape predators (Visse 2007 and references therein). By increasing the apparent size of their hosts, epibionts could also make their hosts more conspicuous to visual predators (Wahl 1989; Xu and Burns 1991; Chiavelli et al. 1993). The hosts may face competition from their epibionts for limiting resources such as food (Kankaala and Eloranta 1987). Many studies have demonstrated that these effects manifest in reduced

zooplankton host fitness (Xu and Burns 1991; Weissman et al. 1993; Visse 2007; Burris and Dam 2014).

The varied negative effects of epibionts on their hosts have led some researchers to use the term “parasite” interchangeably with “epibiont” (e.g. Gouda 2006; López-Téllez et al. 2009; Reda 2011; de Pádua et al. 2013). However, the majority of ecological studies use “parasite” to denote a more invasive interaction (such as reproduction or feeding in/on the host). Besides, unlike parasitism, the effects of epibiosis are often context-dependent, with benefits and disadvantages to both participants driven by biotic (e.g. host abundance, food availability, predation pressure) and abiotic (e.g. salinity, oxygen, pollution) factors. In this thesis, the term “epibiont” will be used to describe attached organisms, irrespective of any apparent benefits and/or trade-offs inherent in the interaction.

## **Background research on ciliate epibionts**

Ciliates are one of the most diverse protist groups and occupy virtually every water body globally (Foissner et al. 2008). These organisms play an important role in the microbial food web of aquatic systems as they are grazers of bacteria and phytoplankton (Michaels and Silver 1988; Sherr and Sherr 1988, 2002; Belviso et al. 1990; Bickel et al. 2012; Safi et al. 2014). Diverse taxa including suctorians, chonotrichs and peritrichs commonly spend part of their life cycle as epibionts on crustacean hosts (Herman and Mihursky 1964; Fenchel 1965, Green 1974; Henebry and Ridgeway 1979; Gilbert and Schröder 2003; Utz 2007).

The earliest studies of ciliate epibionts mainly revolved around distribution and taxonomy (e.g. Corliss and Brough 1965; Green 1965, 1974; Magagnini and Verni 1988). This was then followed by attempts to relate the occurrence and/or seasonality of ciliate epibionts to environmental parameters (e.g. Green 1974; Antipa 1977; Henebry and Ridgeway 1979; Scott and Thune 1986; Xu 1992; Chiavelli et al. 1993; Utz 2003). These studies were field-based, with no experimental evidence of the drivers of epibiosis. Several researchers then began experimentally investigating the effects of epibiosis on host organisms (e.g. Xu and Burns 1991; Weissman et al. 1993; Visse 2007; Burris and Dam 2014).

Xu and Burns (1991) and Burris and Dam (2014) investigated the effects of the peritrich epibionts *Epistylis daphnia* and *Zoothamnium* sp. on the fitness of the calanoid copepods *Boeckella triarticulata* and *Acartia tonsa*, respectively. In both studies, no difference was found

in mortality between well-fed hosts and non-hosts; on the other hand, significantly shorter lifespans were recorded for starving hosts compared with starving non-hosts. Through *in situ* experiments, Weissman et al. (1993) determined that the egg production of the calanoid *A. hudsonica* is negatively correlated to peritrich load (quantity of ciliate zooids per host). Visse (2007) also found that the peritrich *Epistylis* sp. reduced the survivorship of the calanoid *A. bifilosa*.

Ciliate epibionts are frequently found attaching to just one or a few species despite the presence of other species. This might indicate a host density-dependent association, if the preferred host/s also happen to be the most dominant numerically (Valbonesi and Guglielmo 1988; Xu 1992). Alternatively, this attachment to a restricted number of species might be due to host-specificity, whereby host preference is due to reasons other than, or more than just host abundance (eg. the host's carapace texture, moulting frequency, longevity). In this case, disappearance of the host species reflects in the absence of the epibiont, irrespective of high abundance of other species (Henebry and Ridgeway 1979; Xie et al. 2001; Utz and Coats 2005). In cases where the epibiont exerts a negative effect on host fitness, host density dependence and/or specificity also affects community structure through population control of the host species.

Limited studies have considered the biology and ecology of the epibiont itself. Information on the epibiont sheds insight on aspects of its interaction with the host, including seasonal patterns of occurrence, host-epibiont dynamics and host specificity (Hanamura 2000; Utz 2003). Crustacean host biology, such as life cycle and moulting frequency may also be deduced from information about the epibiont it hosts (Utz 2003). To date, only two comprehensive studies exist with information pertaining to the biology and/or ecology of peritrich epibionts (Gilbert and Schröder 2003; Utz 2003).

Gilbert and Schröder (2003) focussed on *Epistylis pygmaeum*. Among other things, these authors investigated the reproduction of *E. pygmaeum* zooids, whether *E. pygmaeum* attaches equally well to different zooplankton species (host-specificity) and whether it attached to specific sites on its hosts (site-specificity). Their findings were that while *E. Pygmaeum* attached to a wide range of species, this epibiont did exhibit selectivity, as it did not attach at all to some other species. Overall, *E. Pygmaeum* exhibited a preference for the eggs rather than the body of various rotifer species, but generally attached to the carapace of cladocerans.

Gilbert and Schröder (2003) also showed for the first time that epibiotic peritrichs (or at least *E. pygmaeum*) can multiply during their non-attached motile stage.

In her PhD thesis, Utz (2003) investigated the formation of *Zoothamnium intermedium* telotrochs (free-swimming, host-seeking stage) and the rate at which they re-attach to a host. Utz (2003) also conducted experiments to determine whether *Z. intermedium* could attach equally well to different stages (juveniles and adults) of its two calanoid copepod hosts, *A. tonsa* and *Eurytemora affinis*, as well as to other members of the Chesapeake Bay zooplankton community. Utz (2003) found that telotroch formation was induced by host mortality and that attachment success of these telotrochs decreased with age. This peritrich attached equally well to copepodite and adult hosts and epibiont growth rates were higher on adults of the pelagic *A. tonsa* compared to the epibenthic *E. affinis*.

Utz (2003) also found that *Z. intermedium* could attach to barnacle nauplii and a harpacticoid copepod (both in the absence of its two host calanoids). This peritrich however, failed to attach to a rotifer species and to inanimate objects. To date, the findings of this PhD research as well as those of Gilbert and Schröder (2003) constitute the bulk of what is known about aspects of the life cycle of epibiotic peritrich ciliates.

### **Study site: the St Lucia Estuary**

The temporarily open/closed St Lucia Estuary (28°23'S, 32°24'E) is a Ramsar Wetland of International Importance and falls within the iSimangaliso Wetland Park, South Africa's first UNESCO World Heritage Site (Begg 1978, Cyrus et al. 2011). With an area of ~ 325 km<sup>2</sup>, St Lucia represents about 80 % of KwaZulu-Natal's total estuarine area and is Africa's largest estuarine lake (Begg 1978; Cyrus and Vivier 2006). Three large but shallow lakes (South Lake, North Lake and False Bay) make up the system (Miranda et al. 2014 and references therein; Figure 1.1). This estuary is governed by cyclical wet and dry phases, with each lasting at least a year and up to 10 years (Begg 1978; Taylor 2006). Even by estuarine standards, St Lucia is very dynamic and experiences extreme conditions exacerbated by past anthropogenic impacts (Begg 1978; Cyrus et al. 2011; Whitfield et al. 2013).

Six rivers feed into St Lucia: the Hluhluwe, Nyalazi, Mpate, Mzinene, Mkhuze and Mfolozi rivers (Whitfield and Taylor 2009). Prior to 1920, the Mfolozi was the biggest contributor of freshwater, with inflow from this river buffering drought symptoms during dry phases in St

Lucia (Begg 1978; Whitfield and Taylor 2009; Whitfield et al. 2013). However, anthropogenic activities including canalisation of the Mfolozi floodplain resulted in the loss of filtering services previously provided by the floodplains and in high sediment loads being carried by Mfolozi water into St Lucia (Begg 1978). The threat posed by sediment loading led to the decision to artificially separate the common Mfolozi-St Lucia mouth in 1952 (Cyrus et al. 2011).

This isolation from Mfolozi deprived St Lucia of an important freshwater source and exacerbated subsequent droughts (Whitfield and Taylor 2009; Cyrus et al. 2011). Attempts have been made to rectify this problem, which include re-connecting St Lucia to Mfolozi by means of a back channel in the late 1960s (Taylor 2013) and a beach spillway in 2012 (Whitfield et al. 2013), the latter of which is also periodically connected to the Indian Ocean. The turbidity problem however persists, particularly during wet phases, with inflow of sediment-laden Mfolozi water. Because St Lucia is also naturally a shallow system (< 2 m depth), turbidity is also caused by wind-induced re-suspension of particulate matter (Carrasco et al. 2007 and references therein).

St Lucia exhibits a reversed salinity gradient, as salinity increases with distance from the estuary mouth (Miranda et al. 2014). The northern sites (North Lake and False Bay) typically experience higher salinities than the southern parts of St Lucia (including Charter's Creek, and sites close to the Mouth; Figure 1.1). This salinity gradient becomes more pronounced during dry phases, with salinities in excess of 100 often being recorded in the northern sites (Grindley 1981; Whitfield and Taylor 2009). Therefore, the temporarily open/closed nature of St Lucia, coupled with the cyclic wet and dry phases as well as sediment loading, result in the St Lucia biota being impacted by fluctuating temperature, salinity and turbidity among other factors. The temporally and spatially dynamic environmental conditions manifest in distinct biotic communities (Grindley 1981, 1982; Carrasco et al. 2010; Carrasco and Perissinotto 2011).

Due to its globally recognised importance, St Lucia is one of the better-studied estuaries in Africa, with studies from research disciplines including hydrology, geomorphology, and ecology (Whitfield and Taylor 2009 and references therein; Perissinotto et al. 2013). This current project was conceived after observing epibionts in zooplankton samples collected from this estuary throughout 2014.

## Zooplankton of St Lucia

The zooplankton community within St Lucia is typically dominated by the mysid *Mesopodopsis africana*, the cyclopoid copepod *Oithona brevicornis* and the calanoid copepods *Acartiella natalensis* and *Pseudodiaptomus stuhlmanni* (Carrasco et al. 2010; Carrasco and Perissinotto 2015; Jones et al. 2016a). During wet phases, freshwater taxa (predominantly cyclopoid copepods and cladocerans) gain dominance, while the abundance of all the resident estuarine species declines markedly (Jones et al. 2016a). However, *P. stuhlmanni* is more tolerant of low salinity (including limnetic) and high turbidity ( $\geq 1000$  NTU) (Grindley 1981; Jones et al. 2015 and references therein; Jones et al. 2016a). Unlike the other zooplankton species, this calanoid appears to be present year round in St Lucia, irrespective of condition (S. Jones pers. observ. from 2013–2017).

*Pseudodiaptomus stuhlmanni* also appears to be the preferred host of unidentified epibiotic peritrich ciliates, as observations of field-collected samples have revealed these peritrichs attached to this calanoid despite the availability of other species (Jones et al. 2016a; also see Supplementary material A).

## Aim of this study

The focus of this thesis is epibiosis involving *P. stuhlmanni* and the peritrich ciliates sampled within the St Lucia Estuary (see Supplementary material B for the various stations sampled). The broad aim is to tackle epibiosis from different levels of biological organisation. The perspective of both the epibiont and host will be considered and this epibiosis will be observationally and experimentally related to environmental conditions. Specifically, the impacts of salinity, temperature and turbidity on the epibionts will be examined. The overarching hypothesis is that the peritrich epibionts will have an effect on their hosts but that the effects will be modulated by environmental conditions.

This is the first comprehensive study concerning sessilid peritrichs and zooplankton hosts in Africa. Existing studies in this region have mainly been of a taxonomic nature and have dealt with mobilid peritrichs or sessilids on fish and other non-zooplanktonic hosts (e.g. Green 1965; Van As and Basson 1984; Viljoen and Van As 1985; Basson and Van As 1991, 1993; Gouda 2006; West et al. 2016).

**Objectives of this study:**

- A. Provide the first account and initial morphological characterisation of epibiotic peritrichs in the St Lucia Estuary using live observations, the protargol staining technique and scanning electron microscopy (Chapter 1).
- B. Experimentally test 1) the specificity of the peritrichs for the copepod *P. stuhlmanni*, 2) whether the epibiotic relationship is host density dependent and 3) the effect of the peritrichs on the survivorship of *P. stuhlmanni*. This work is detailed in Chapter 2.
- C. Determine the effects of the epibionts on the health of *P. stuhlmanni* at the sub-organismal level using the RNA:DNA ratio and RNA content (Chapter 3).
- D. Experimentally test the relationship between the peritrichs and turbidity (organically rich and inorganic), salinity and temperature (Chapters 4 and 5).
- E. Determine the dynamics of the peritrichs and *P. stuhlmanni* and relate these to environmental conditions over a one-year period in the St Lucia Estuary (Chapter 6).

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## A first record and initial morphological characterisation of the epibiotic peritrich *Epistylis* sp. (Ciliophora: Peritrichia) in the St Lucia Estuary, South Africa

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### ABSTRACT

A peritrich ciliate was found living as an epibiont on the copepod *Pseudodiaptomus stuhlmanni* in the St Lucia Estuary, South Africa. Live observations, protargol staining and scanning electron microscopy (SEM) were used in the initial morphological identification of this peritrich. Based on diagnostic features including a rigid attachment stalk and everted peristomial lip, this epibiont was identified as belonging to the genus *Epistylis* sp. Ehrenberg, 1830 within the family Epistylididae Kahl, 1933. Further work, especially genetic-based analysis, is needed for a species-level identification of this population of *Epistylis* sp.

**Keywords:** ciliate, copepod host, epibiosis, identification

### INTRODUCTION

The subclass Peritrichia comprise a large assemblage of ciliates characterised by specialized oral cilia and somatic cilia reduced to a trochal band (Utz et al. 2010). Sessilida and Mobilida are the orders belonging to subclass Peritrichia (Utz et al. 2010; Sun et al. 2016 and references therein) and while Mobilids are exclusively symbiotic, Sessilid peritrichs may be free-living and in symbiotic associations (Liu and Gong 2012). Most symbiotic sessilids possess a stalk with which they attach to their hosts (Wang et al. 2017). Epibiotic sessilids have been found in zooplankton samples collected in the St Lucia Estuary, Africa's largest estuarine lake and a RAMSAR wetland of International Importance (Figure 1.1). These epibionts have only been observed attached to the copepod *Pseudodiaptomus stuhlmanni*, which is among the numerically dominant zooplankton species in St Lucia. To my knowledge, this is the first record of ciliate epibionts attaching to crustaceans in KwaZulu-Natal.

Traditionally, live observations were used to identify epibiotic ciliates to genus and even species level (e.g. Green 1965). The problem with this approach is that only a limited number of features can be observed from live specimens. Cytological techniques, such as the protargol silver staining method, reveal characters otherwise invisible under normal light microscopy, such as infraciliature and macro- and micro-nuclei (Zagon and Small 1970; Montagnes and Lynn 1987).

A growing number of researchers are also using scanning electron microscopy (SEM) to view features such as cell topology and characteristics of the scopula and peristome that aid in accurate identification (Utz 2003; Ma and Overstreet 2006; Wang et al. 2017). In this chapter, live observations, the protargol staining procedure and SEM are used in the initial morphological characterisation of the St Lucia peritrich epibionts.

## **MATERIALS AND METHODS**

### **Sample collection**

Samples were collected from Charter's Creek and Dredger Harbour in the St Lucia Estuary (Figure 1.1) between 2015 and 2017. The zooplankton was sampled using either a hand-held 100 µm mesh seive or a hyperbenthic sled fitted with 100 µm mesh net. The samples were thereafter either taken to the laboratory alive for live observations or preserved in 5 % Bouin's fixative (to be used in protargol staining) or 2.5 % glutaraldehyde (for SEM preparation).

### **Protargol staining**

Specimens preserved in 5 % Bouin's fixative were rinsed with distilled water and thereafter stained using the protargol method (Montagnes and Lynn 1987) but modified as described by Utz et al. (2008 [see Supplementary material no. 1.1]).

### **Scanning Electron Microscopy**

Epibiont-hosting *P. stuhlmanni* individuals fixed with 2.5 % glutaraldehyde were rinsed in distilled water to remove the fixative and thereafter dehydrated in a graded ethanol series. The specimens were then critical-point dried using carbon dioxide, coated with a gold-palladium alloy and visualized using a Field Emission Scanning Electron Microscope (FE-SEM, Zeiss Gemini Ultra Plus).

### **Morphometric data**

A Nikon Eclipse 80i microscope fitted with a Nikon Digital Sight camera and NIS Elements D3.0 software were used to view, measure and photograph the specimens. The morphological features of live and protargol-stained peritrichs were measured using bright field and differential interference contrast at magnifications up to 100 ×. Values are reported as mean ± standard deviation.

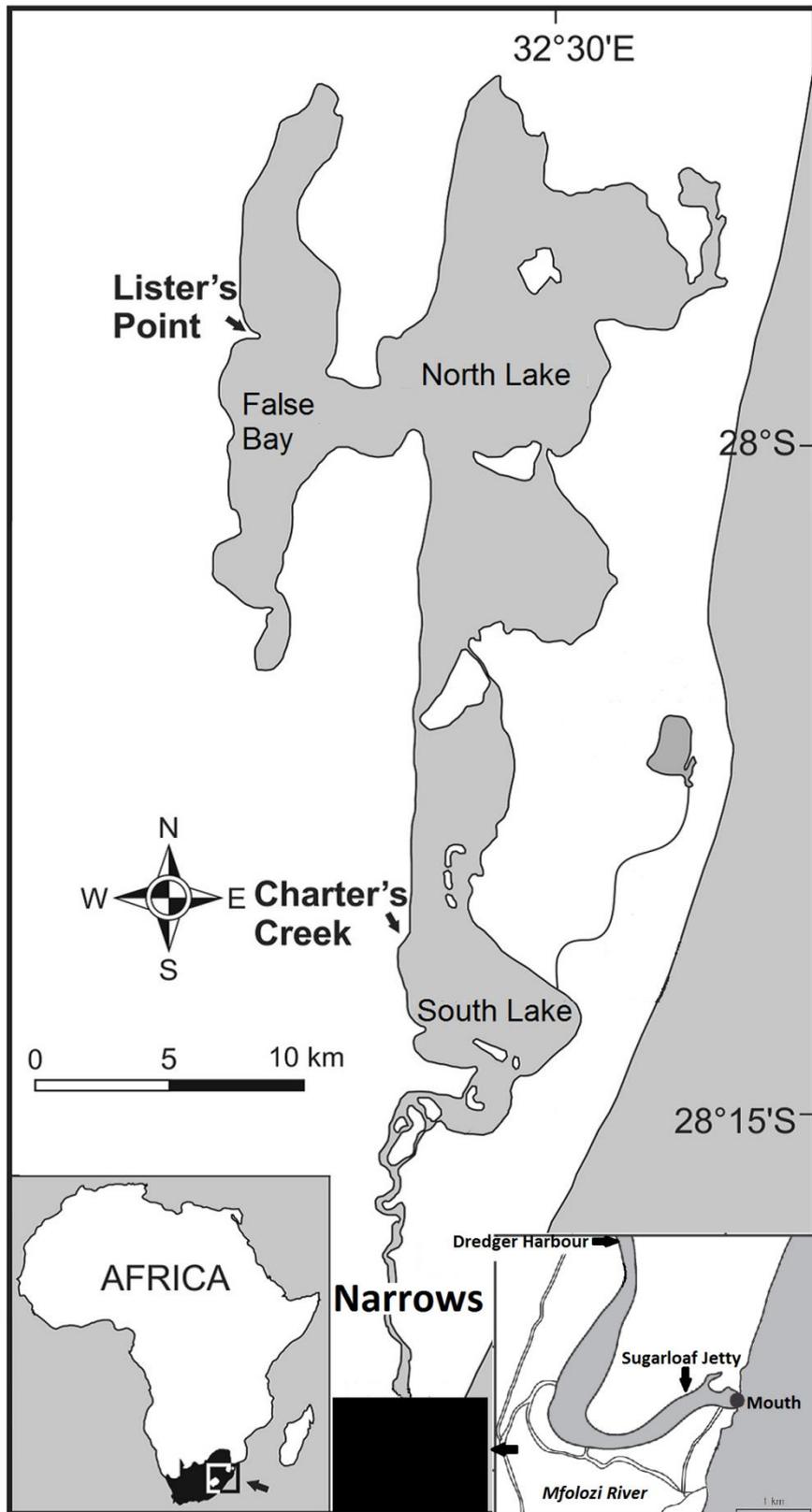


Figure 1.1: Map of the St Lucia Estuary. Arrows point to all the stations sampled during the course of this research. Adapted from Peer et al. (2014).

## RESULTS

### Morphology

This peritrich had an everted peristomial lip, a rigid attachment stalk and less than two ciliary rows, all of which characterize the genus *Epistylis* sp. Ehrenberg, 1830 within the family Epistylididae Kahl, 1933 (Figure 1.2 A–K). Expanded zooids had an elongated inverted bell-shape and there was a slight constriction beneath the peristomial lip (Figure 1.3 B). Epibionts were only observed on adult hosts, which are ~ 1mm in size. The length and width of live expanded zooids was  $37.3 \pm 9.01$  and  $25.1 \pm 5.08$   $\mu\text{m}$ , respectively. The length and width of live contracted zooids was  $30.7 \pm 6.02$   $\mu\text{m}$  and  $28.4 \pm 4.99$   $\mu\text{m}$ , respectively (Table 1.1). Most zooids expanded during the fixation process. Therefore, the length and width of expanded protargol-impregnated zooids is reported; the length was  $33.6 \pm 6.84$   $\mu\text{m}$  and the width was  $27.5 \pm 4.46$   $\mu\text{m}$  (Table 1.2).

The protargol stain revealed a C-shaped macronucleus positioned either transversely or longitudinally within the zooid (Figures 1.2 H–J and 1.3 B). The longitudinal position was only observed once, on a copepod that otherwise hosted zooids with transversely positioned macronuclei (Figure 1.2 J). The spherical micronucleus was positioned close to the macronucleus (Figure 1.2 I and J).

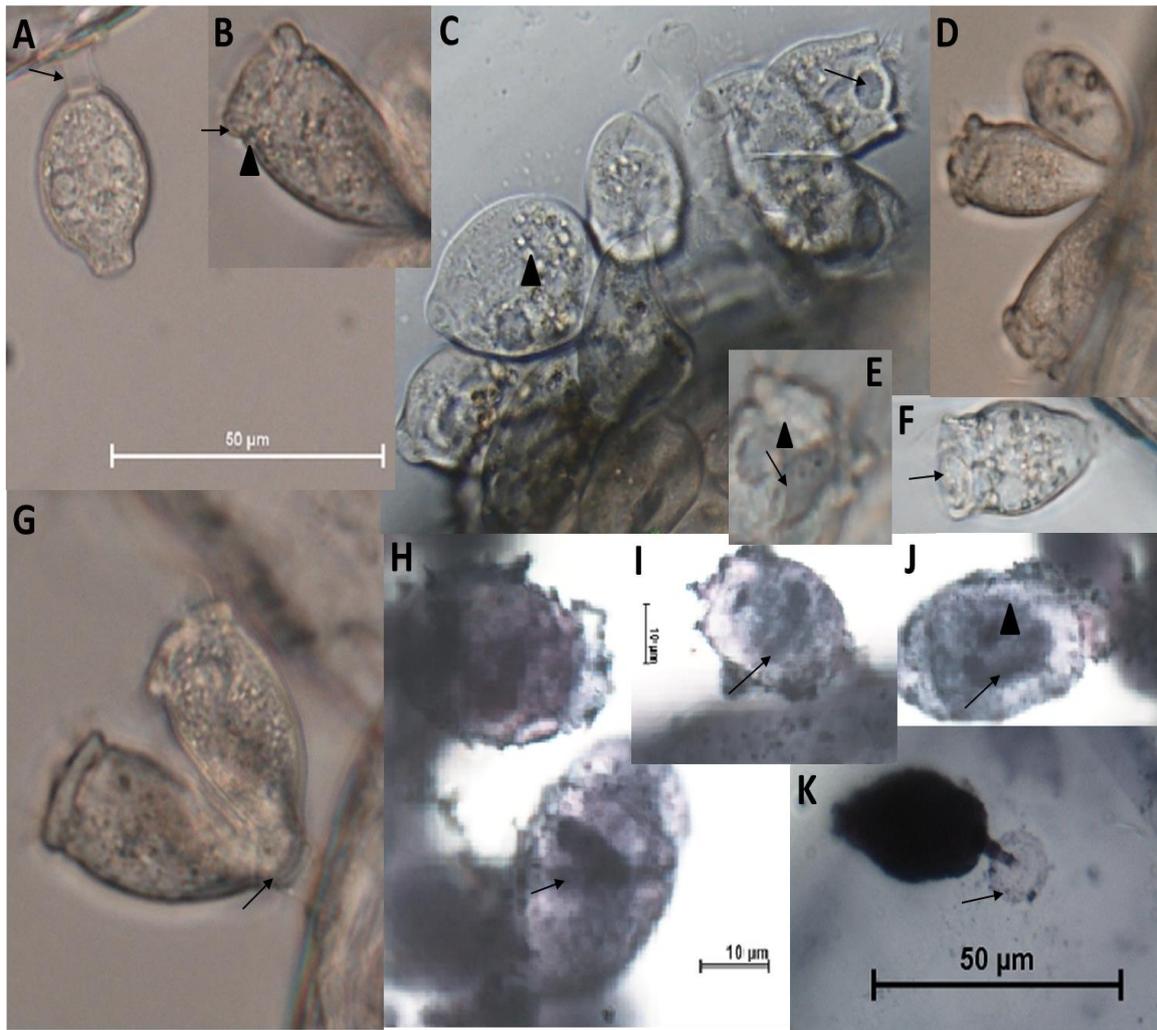


Figure 1.2: Morphology of *Epistylis* sp. from live cells (A–G) and after protargol impregnation (H–K). First and second feature denoted by an arrow and arrowhead, respectively. A- contracted zooid also showing the short and empty attachment stalk. B- expanded zooid showing the everted peristomial lip and a slight constriction below the peristomial lip. C- colony showing the contractile vacuole and food vacuoles inside the zooids. D- colony with two expanded and one contracted zooid. E- expanded zooid showing the infundibulum and contractile vacuole. F- expanded zooid showing the peristome region. G- colony comprised of two zooids as well as showing the branching of the stalk. H and I- zooids showing the transversely positioned C-shaped macronuclei. J- zooid showing a longitudinally positioned C-shaped macronucleus and a micronucleus positioned close to it. K- specimen showing the attachment disk on the exoskeleton of the copepod *P. stuhlmanni*. A,B, D, G & I–K collected from Dredger Harbour and C, E, F & H collected from Charter's Creek within the St Lucia Estuary.

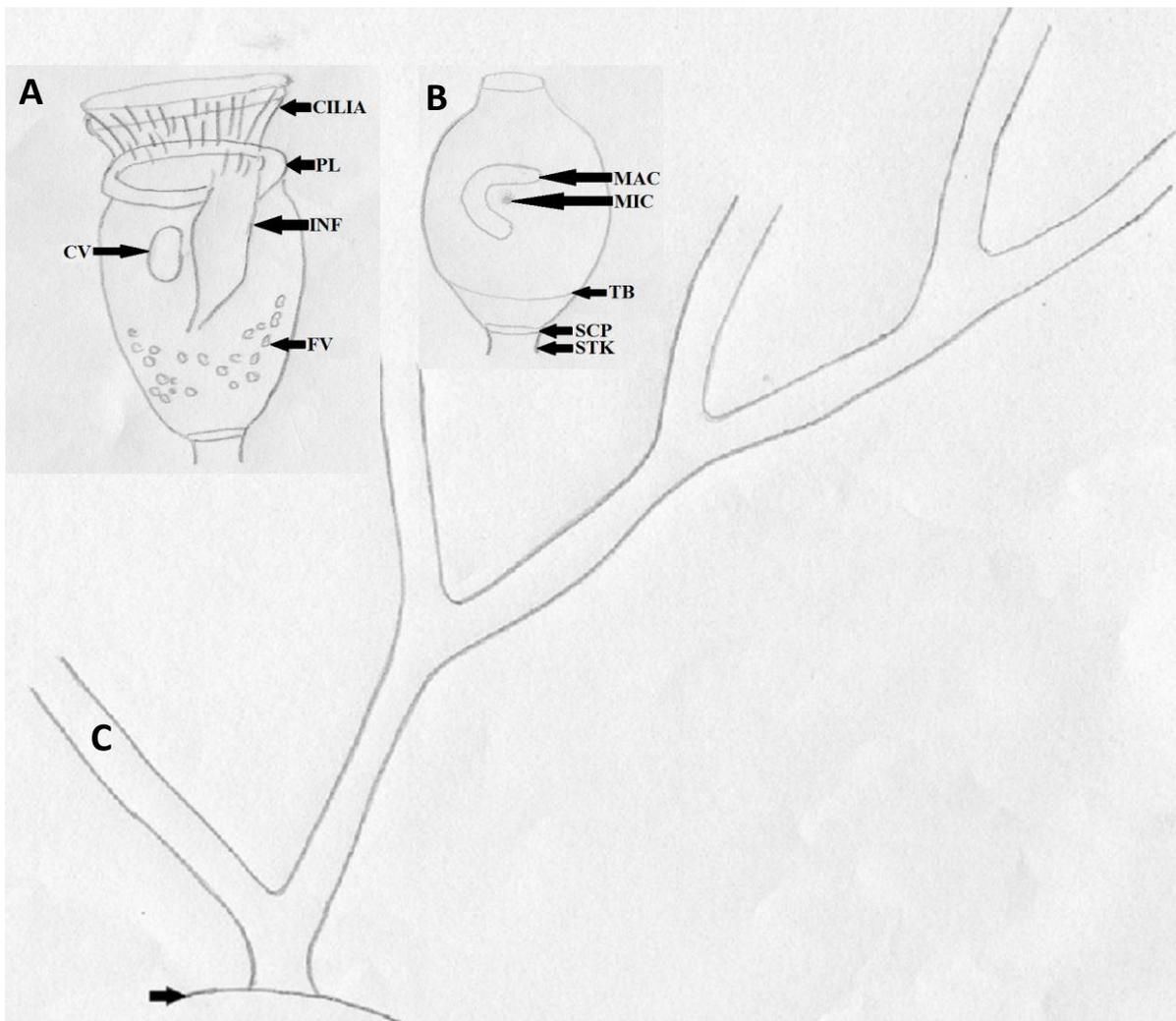


Figure 1.3: Drawings of *Epistylis* sp. A and B- internal features observed from live and protargol-stained specimens. A- shape of expanded zooid. Also showing cilia in the peristome region, peristomial lip (PL), infundibulum (INF), contractile vacuole (CV) and food vacuoles (FV). B- shape of contracted zooid as well as the macro- and micro-nucleus (MAC and MIC), telotroch band (TB), scopula (SCP), and stalk (STK). C- dichotomous branching pattern of the stalk, with alternate branches terminating at the same height; note the height and thickness of the basal stalk relative to its branches; arrow points to *P. stuhlmanni* exoskeleton.

Table 1.1: Morphometric characteristics of live *Epistylis* sp. attached to adults of the copepod *P. stuhlmanni* (which is ~ 1 mm) from the St Lucia Estuary (collection site: Dredger Harbour; n = 23 in all cases).

<b>Character</b>	<b>Min (µm)</b>	<b>Max (µm)</b>	<b>Mean (µm)</b>	<b>SD (µm)</b>	<b>CV (%)</b>
Zoid length extended	27.4	50.6	37.3	9.01	24.2
Zoid length contracted	21.1	44.2	30.7	6.02	19.6
Zoid width extended	18.3	34.1	25.1	5.08	20.2
Zoid width contracted	18.1	37.2	28.4	4.99	17.6
Peristomial lip width	15.3	25.2	19.3	2.87	14.9
Peristomial lip height	1.89	3.68	2.84	0.554	19.5
Basal stalk width	3.84	5.11	4.39	0.329	7.5
Secondary stalk width	2.36	5.16	3.44	0.815	24
Distance from stalk base to first branching point	2.08	4.62	3.35	0.708	21.1
Zoid number per basal stalk	0	28	8.65	6.76	78.2

Table 1.2: Morphometric characteristics of protargol-stained *Epistylis* sp. attached to the copepod *P. stuhlmanni* from the St Lucia Estuary (collection site: Dredger Harbour; n = 23 in all cases).

<b>Character</b>	<b>Min (µm)</b>	<b>Max (µm)</b>	<b>Mean (µm)</b>	<b>SD (µm)</b>	<b>CV (%)</b>
Zoid length extended	23.1	51.3	33.6	6.84	20.4
Zoid width extended	19.3	37.6	27.5	4.46	16
Peristomial lip width	9.35	24.6	17.4	3.72	21.4
Peristomial lip height	1.76	3.56	2.64	0.485	18.4
Basal stalk width	3.7	5.23	4.42	0.364	8.2
Distance from stalk base to first branching point	1.9	7.34	4.01	1.64	40.9
Secondary stalk width	2.18	4.87	3.24	0.827	25.5
Zoid number per basal stalk	0	32	14.9	10.9	73
Scopula width	3.48	5.91	4.75	0.719	15.1

The SEM images revealed  $10.4 \pm 1.08$  pellicular striations that circle the peristomial lip in a zig-zag pattern and  $59.1 \pm 7.88$  parallel pellicular striations from just below the peristomial lip to the scopula. Pellicular pores were spaced irregularly between the striations all around the zooids (Figure 1.4 A–D).

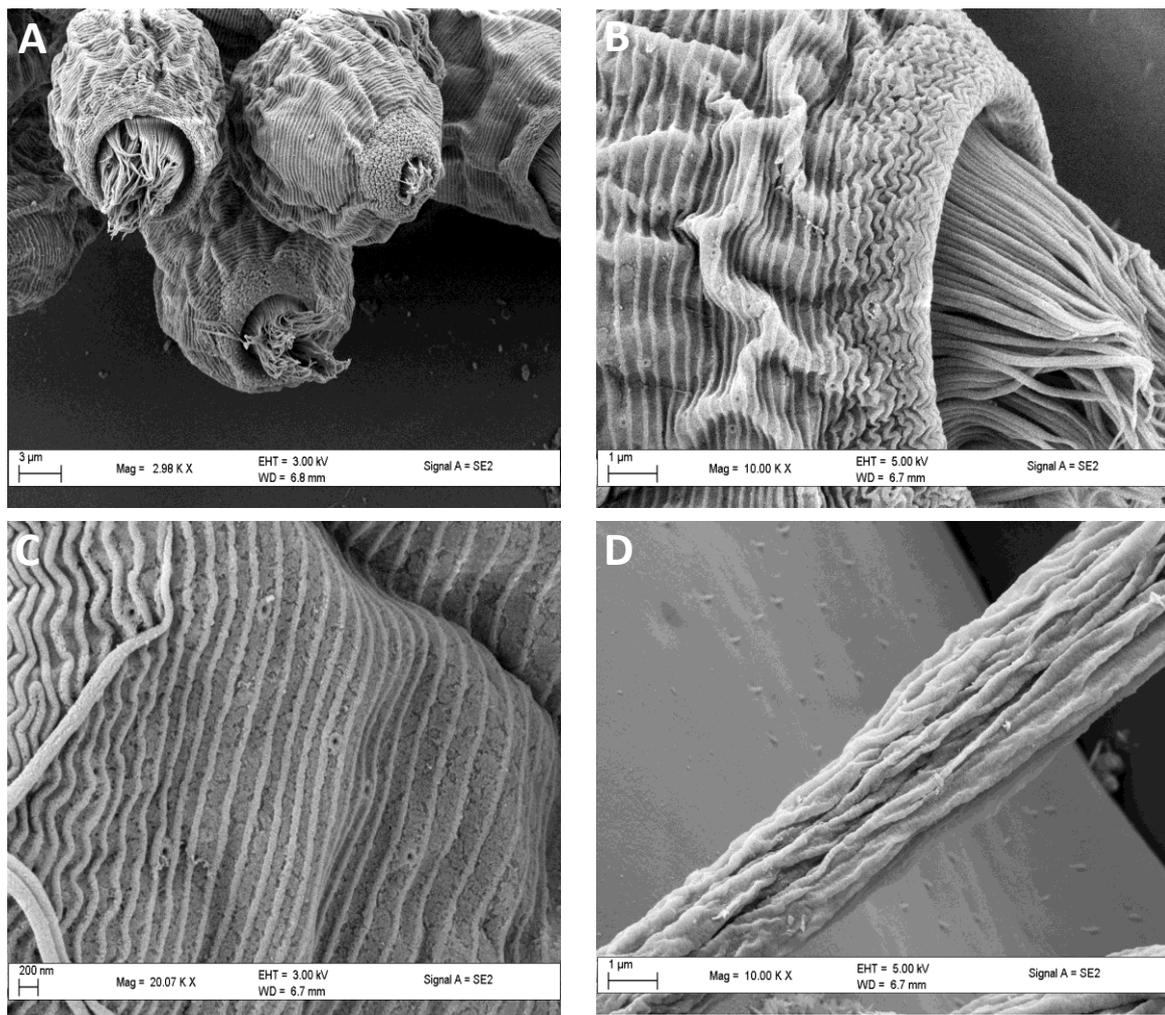


Figure 1.4: SEM micrographs of *Epistylis* sp. A- oral view of expanded and contracted zooids. Note the transverse zig-zag pellicular striations on pellicle in the peristomial lip area and transverse parallel striations on pellicle below the peristome area. B- close-up of the transverse pellicular striations. C- pellicular pores distributed unevenly between the transverse striations. D- thickness and texture of a secondary stalk. A and C collected from Dredger Harbour; B and D collected from Charter's Creek.

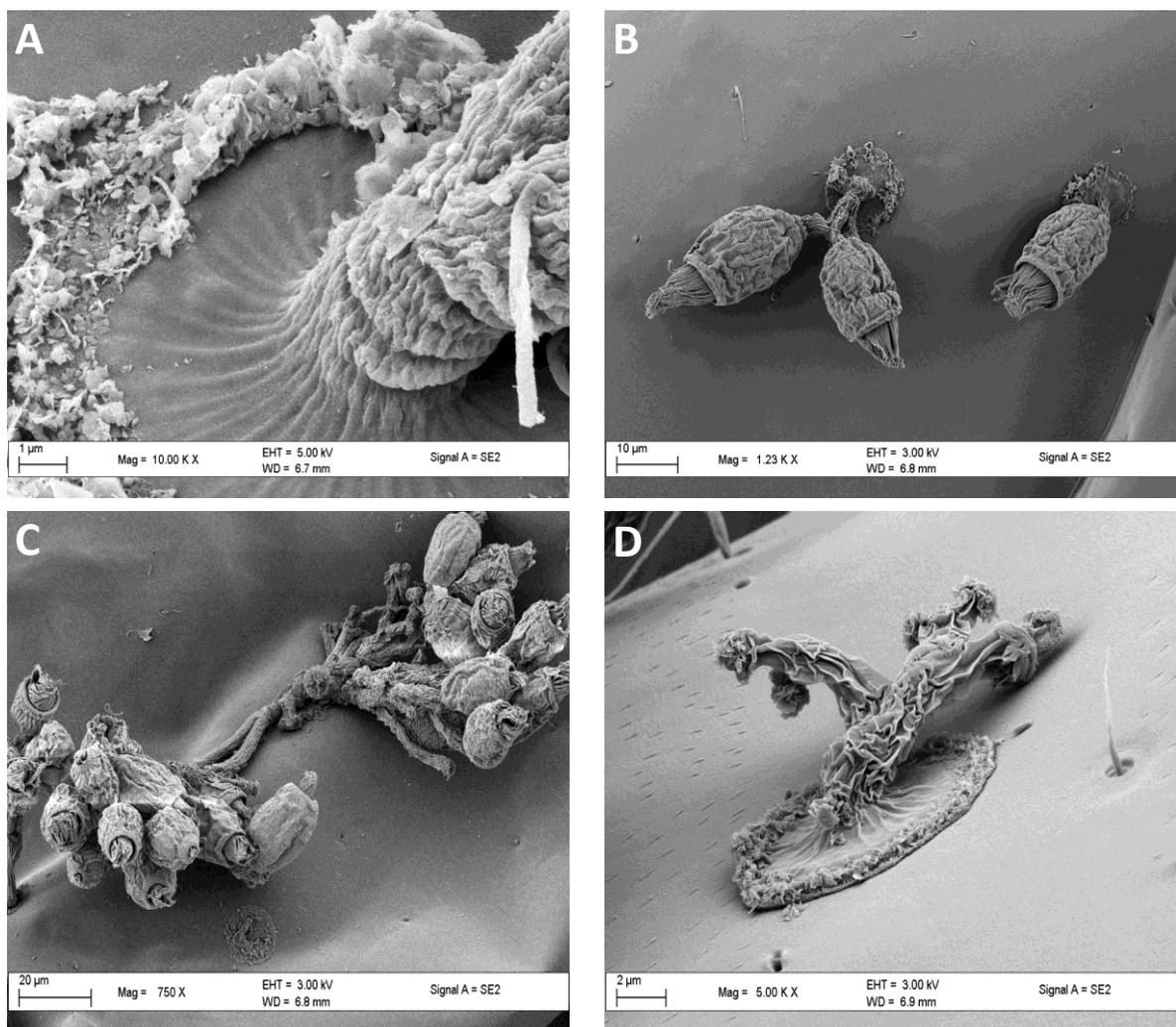


Figure 1.5: SEM micrographs of *Epistylis* sp. A- attachment disk and basal stalk on exoskeleton of the copepod *P. stuhlmanni*. Note transverse ridges around the basal stalk. B- basal stalk hosting a colony of two zooids and a basal stalk directly supporting a single zooid. C- large colony with multiple branches and ~ 20 zooids. Note that some stalks have no attached zooids. D- a branched stalk with no attached zooids. A, C and D collected from Charter's Creek; B collected from Dredger Harbour.

The stalk was thick, rigid, non-contractile and hollow (Figure 1.2 A and G; Figure 1.4 D) and the SEM micrographs revealed transverse ridges encircling the surface of the basal stalk (Figure 1.5 A). The basal stalk was usually the thickest (Tables 1.1 and 1.2; Figures 1.3 C and 1.5 D) and either directly supported zooids, or branched off dichotomously to form multiple secondary stalks (Figure 1.5 B and C). The number of zooids per colony ranged from 2 to 28 for live specimens and from 2 to 32 for protargol-stained specimens. Colonies with more than ~12 zooids typically had stalks branching off from the secondary stalks. Some stalks supported one zooid, or had no attached zooids at all (Tables 1.1 and 1.2; Figure 1.5 B–D). The attachment disk was circular in shape (Figure 1.2 K; Figure 1.5 A, B and D; Figure 1.6 A–C). The SEM

images also revealed lesions and bacteria on *P. stuhlmanni*'s exoskeleton around the stalk attachment area (Figure 1.6 A and B), lifting of the attachment disk from the exoskeleton of *P. stuhlmanni* (Figure 1.6 C), and white patches on *P. stuhlmanni* where stalks had either been attached or had been attaching prior to fixation (Figure 1.6 D).

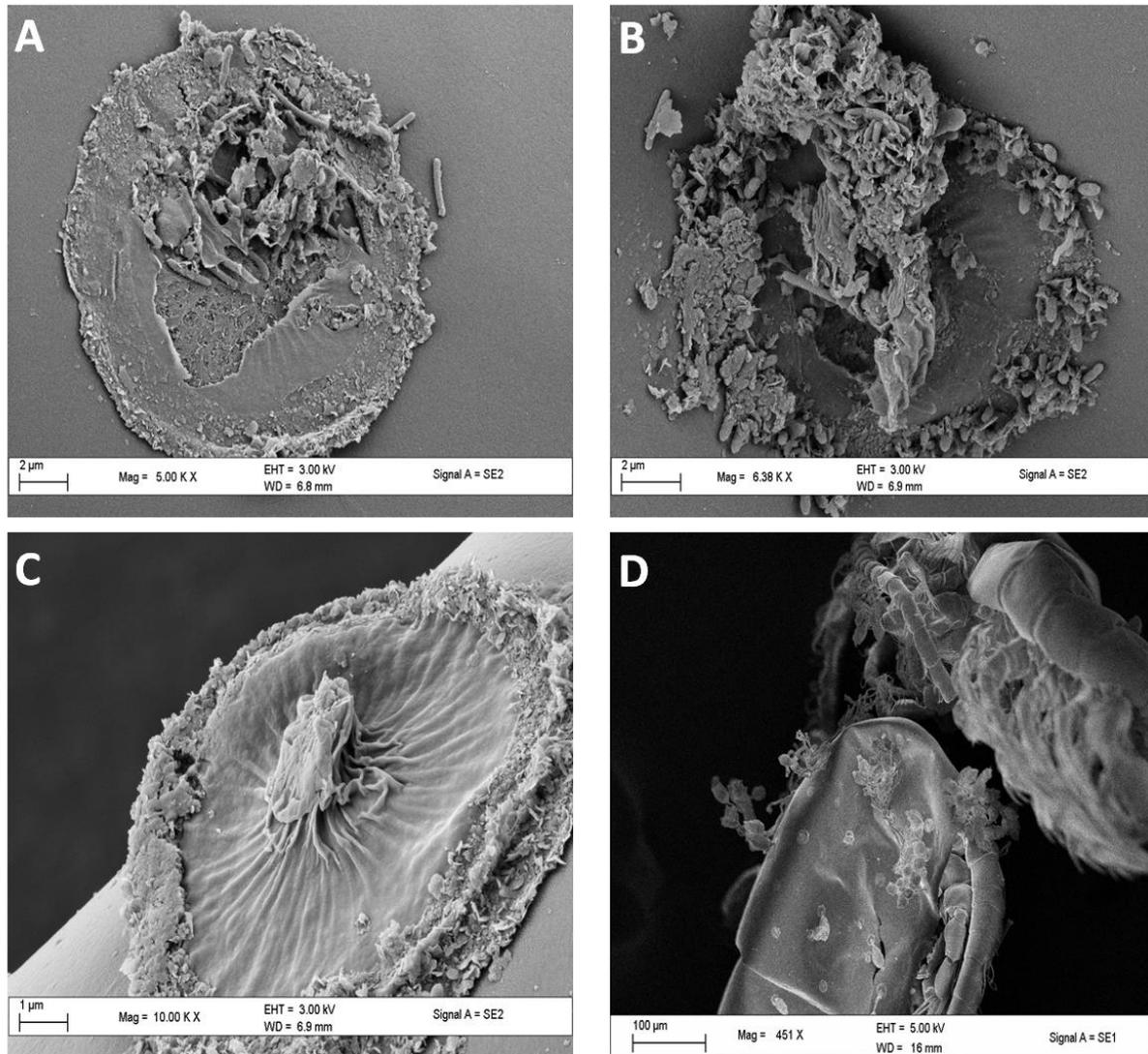


Figure 1.6: SEM micrographs of *Epistylis* sp. A and B- attachment disk with bacteria and visible damage to the exoskeleton of *P. stuhlmanni*. C- Attachment disk lifting on one side. D- *P. stuhlmanni* exoskeleton with attached *Epistylis* sp. colonies. Note the white spots where *Epistylis* sp. stalks had either detached or had been in the attachment process prior to fixation. Collected from Dredger Harbour.

### **Taxonomic summary**

Class Oligohymenophorea de Puytorac et al., 1974

Subclass Peritrichia Stein, 1859

Order Sessilida Kahl, 1933

Family Epistylididae Kahl, 1933

Genus *Epistylis* Ehrenberg, 1830

### **DISCUSSION**

Similar to other peritrichs, the descriptions of most species in the genus *Epistylis* have been based on live observation (e.g. Fauré-Fremiet 1930; Lom 1964). Subsequent studies (e.g. Bauer-Nebelsick et al. 1996; Cho and Shin 2003, Utz 2007; Qi et al. 2009) have also used silver staining techniques. The stain is intended to reveal internal structures including the macro- and micronucleus as well as the infraciliature, which aid in identification to species level. In this study, although the macro- and micro-nuclei were made visible by the protargol stain, the infundibular polykineties were not, even after multiple attempts and slight modifications of some steps (Supplementary material 1.2). This prevented the identification to species level. As some genetic studies have found that the genus *Epistylis* is not monophyletic (Utz et al. 2010; Sun et al. 2016; Wang et al. 2017), DNA sequencing of the St Lucia population before species-level identification is recommended.

Many studies have reported negative impacts of peritrich epibionts on the fitness of their zooplankton hosts (Weissman et al. 1993; Gilbert and Schröder 2003; Visse 2007). In this study, SEM images revealed mechanical damage of the copepod exoskeleton where *Epistylis* sp. stalks had been attached. Turner et al. (1979) also found that *Epistylis* sp. caused lesions on the carapace of the copepod *Acartia tonsa* in Escambia Bay, Florida. In this study and in Turner et al. (1979), bacteria were found surrounding the damaged areas. Potential impacts of *Epistylis* sp. on the fitness of *P. stuhlmanni* are detailed in Chapters 2 and 3.

An unusual observation from the St Lucia *Epistylis* sp. population has been the association of these epibionts with very small (~ 10 µm) organisms, which actively swim inside the carcasses of *P. stuhlmanni* and another dominant calanoid in St Lucia, *Acartiella natalensis*. These organisms have now been observed twice, the first time in samples collected in the Narrows region of St Lucia in March 2014, following a flood event, and again in samples collected at Dredger Harbour in September 2017 (Supplementary material 1.3). Both occasions coincided

with zooplankton samples that had a high prevalence of *Epistylis* sp. (attached only to *P. stuhlmanni*).

The organisms observed in the copepod carcasses somewhat resemble the histophagous tomite stage of apostome ciliates (see Ohtsuka et al. 2011). Apostomes also have an attached stage, which has never been observed in any of the St Lucia zooplankton. It is possible that there are apostome ciliates that attach to a different host such as benthic crustaceans, fish or molluscs within St Lucia and that their tomite stage invades crustacean carcasses. Their association with *Epistylis* sp. nonetheless warrants more attention.

In summary, this study aimed to report the occurrence and provide an initial morphological characterisation of a peritrich epibiont found attached to a calanoid copepod in the St Lucia Estuary. Based on live observations, scanning electron microscopy and some features revealed by the protargol stain, this peritrich has been identified as *Epistylis* sp. However, more features need to be described in order to get a species-level identification. Due to the conflicting results obtained from some genetic analyses of species in this genus, identification to species level will need to be verified with DNA sequencing.

## **ACKNOWLEDGEMENTS**

I am very grateful to Dr Laura Utz from Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) for teaching me the protargol staining technique and for assisting with the identification of *Epistylis* sp. I also thank Marcos Pereira for his support during my visit at PUCRS. I thank Subashen Naidu and Vishal Bharuth from the Microscopy and Micro-analysis Unit at the University of KwaZulu-Natal for assisting with scanning electron microscopy.

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## ***Epistylis* sp. host specificity, host density dependence and effects on the survivorship of the calanoid *Pseudodiaptomus stuhlmanni* in the St Lucia Estuary, South Africa**

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### **ABSTRACT**

The peritrich epibiont *Epistylis* sp. is commonly found attached to the dominant copepod *Pseudodiaptomus stuhlmanni* in the St Lucia Estuary, South Africa. Experiments were conducted, aimed at determining the host specificity and host density dependence of *Epistylis* sp.; and the relationship between this epibiont and the survivorship of *P. stuhlmanni*. Mixed culture experiments with the copepods *P. stuhlmanni*, *Acartiella natalensis* and *Oithona brevicornis* resulted in the exclusive attachment of *Epistylis* sp. to *P. stuhlmanni*. In contrast, no zooplankton hosted *Epistylis* sp. in mixed cultures with *P. stuhlmanni* and the mysid *Mesopodopsis africana*. Monoculture experiments with each of the above species also resulted in attachment only to *P. stuhlmanni*. Prevalence was significantly higher in *P. stuhlmanni* adults when compared with copepodites. A positive relationship was observed between epibiont prevalence and *P. stuhlmanni* density. High epibiont cover was associated with low survivorship of *P. stuhlmanni*. Individuals with a low epibiont cover had a higher survivorship, which was not statistically different from that of non-hosting individuals. These results suggest that *Epistylis* sp. has the potential to shape the zooplankton community structure and to influence the pelagic food web of the St Lucia Estuary.

**Keywords:** epibiosis; estuaries; peritrich ciliates; zooplankton community structure

### **INTRODUCTION**

Epibiosis is a symbiotic association of two organisms in which one, the epibiont, spends the sessile stage of its life attached to the surface of the other, termed the basibiont or host (Wahl 1989; Threlkeld et al. 1993). Epibiosis involving ciliated protozoa and zooplankton is widespread in aquatic environments (Henebry and Ridgeway 1979; Green and Shiel 2000; Fernandez-Leboranz et al. 2005; Bielecka and Boehnke 2014). Attachment to a constantly

moving substrate is beneficial to epibiotic ciliates, as it facilitates dispersion and gene flow and also increases encounter rates with food at little or no energetic cost to the epibiont (Regali-Selegim and Godinho 2004; Bickel et al. 2012).

Although epibiosis has been traditionally described as a commensal relationship, many studies have documented detrimental effects on zooplankton hosts (Gilbert and Schröder 2003; Visse 2007; Bickel et al. 2012; Bielecka and Boehnke 2014). For instance, by increasing burden and interfering with motility, epibiotic ciliates have been shown to decrease the feeding efficiency and increase the susceptibility of their zooplankton hosts to predation (Visse 2007 and references therein). Epibionts may also directly compete with the zooplankton for food, if the size range of ingestible particles overlaps (Kankaala and Eloranta 1987).

*Epistylis* spp. are stalked, colonial peritrich ciliates that have been recorded attached to zooplankton over a wide geographic range, including Chesapeake Bay in North America, the Seine Estuary in France and Lake Velachery in India (Utz and Coats 2005; Rajabunizal and Ramanibai 2011; Souissi et al. 2013). *Epistylis* sp. has now also been found on the zooplankton of the St Lucia Estuary, Africa's largest estuarine lake and a Ramsar Wetland of International Importance (Figure 1.1). Although the zooplankton community of St Lucia is usually dominated by four species (Carrasco and Perissinotto 2015), inspection of zooplankton samples shows that irrespective of the species composition, *Epistylis* spp. attaches exclusively to the calanoid copepod *Pseudodiaptomus stuhlmanni* (Jones et al. 2016a).

Several other studies have documented the tendency of epibionts including *Epistylis* spp. to attach to only one or two species, even in the presence of other zooplankton species. For instance, Xie et al. (2001) found that *Epistylis lacustris* in Crystal Lake, Wisconsin attached mainly to the copepod *Leptodiaptomus minutus* although there were six other available species. Similarly, Utz and Coats (2005) documented the exclusive attachment of *Epistylis* sp. to the calanoid *Acartia tonsa* despite the co-dominance of another calanoid, *Eurytemora affinis* in Chesapeake Bay.

Because epibionts may negatively affect their hosts, epibiont host selectivity has the potential to shape community structure by controlling the population dynamics of the host (Skovgaard and Saiz 2006). Herman and Mihursky (1964) attributed the progressive replacement of *A. tonsa* by *A. clausi* in the Patuxent River Estuary to the strong selection of the former species by the peritrich *Zoothamnium* sp. Weissman et al. (1993) found that epibionts reduced the

survivorship of *A. hudsonica*, thus contributing to the seasonality of this calanoid in the Stony Brook Harbor, Long Island Sound. At St Lucia, control of *P. stuhlmanni* by *Epistylis* sp. may also have trophic-level impacts, as this copepod is a key prey item for planktivorous and benthic fish within the system (Grindley 1982; Nhleko et al. 2012; Peer et al. 2013; Dyer et al. 2015). This study therefore aims to verify: (1) the host specificity of *Epistylis* sp.; (2) the relationship between host density and epibiont prevalence (percentage of population hosting epibionts); and (3) the relationship between epibiont cover (percentage of host body covered) and the survivorship of *P. stuhlmanni*.

## MATERIALS AND METHODS

### Sample collection

Daytime zooplankton samples were collected at Charter's Creek and the Narrows in St Lucia (Figure 1.1) in 2014 and 2015 using a 100 µm mesh D-net mounted on a hyperbenthic sled. All material collected in the cod end of the net was emptied into 20 L buckets filled with water collected at the same site, and transported to the laboratory. All experiments were performed in a controlled temperature room ( $21 \pm 1^\circ\text{C}$ ) under a 12:12 light:dark regime and only healthy (active) individuals were used.

### Zooplankton of the St Lucia Estuary

In mesohaline and polyhaline conditions, the zooplankton community mainly comprise the mysid *Mesopodopsis africana*, the cyclopoid copepod *Oithona brevicornis* and the calanoid copepods *Acartiella natalensis* and *Pseudodiaptomus stuhlmanni* (Carrasco and Perissinotto 2015). At the commencement of the study, *M. africana* was either absent or occurred in low densities. Therefore, the copepods were used in one set of experiments and *M. africana* was later used with *P. stuhlmanni* in another set. St Lucia estuarine water was filtered through a 55 µm mesh in order to exclude other grazers and this water was used in all experiments.

### *Epistylis* sp. host specificity and host epibiont density dependence

Host moulting or mortality triggers transformation of the attached peritrich zooids into free-swimming telotroch zooids, which then disperse in search of new hosts (Clamp 1973; Utz and Coats 2008). To ascertain the host specificity of the St Lucia *Epistylis* sp., heavily infested (> 40% body covered by *Epistylis* sp.) *P. stuhlmanni* were isolated under a Kyowa SDZ dissecting microscope (40 ×) and placed in petri-dishes with a drop of the 55-µm filtered estuarine water. The exact number of zooids on the hosting copepods could not be counted without stressing

both the host and the epibiont; however, for each experiment an attempt was made to standardize by using similarly sized adults with a similar percentage body cover across replicates and treatments. A pair of fine-tipped forceps was then used to crush the cephalosome of these copepods, thus killing them following Bickel et al. (2012). The petri-dishes were then filled with 50 mL of the 55- $\mu\text{m}$  filtered estuarine water. Zooid detachment was observed within the first hour of host mortality.

Epibiont attachment success may be host size dependent, as frequent encounters with larger organisms provide more attachment opportunities (Regali-Selegim and Godinho 2004). To control for this potential bias, monocultures with adult zooplankton were set up such that in each petri-dish 50 individuals of the smaller-sized species, viz. *O. brevicornis* and *A. natalensis* (both < 700  $\mu\text{m}$  length), 25 *P. stuhlmanni* (~1 mm length) and 4 *M. africana* (~1 cm length) were respectively exposed to one heavily infested, dead *P. stuhlmanni* individual. The same experiment was repeated with a mixed culture. In each petridish, 8 *P. stuhlmanni*, 12 *A. natalensis* and 12 *O. brevicornis* (n = 32) were exposed to one dead, heavily infested *P. stuhlmanni* individual. In another set of mixed culture experiments, 3 *M. africana* and 10 *P. stuhlmanni* were similarly exposed. To reduce evaporative water loss, the petri-dishes were covered during the experiments. Preliminary experiments showed that *A. natalensis* and *M. africana* died if exposed for a long time (24 hours). Therefore, a 6-hour exposure time was chosen for these experiments following Utz and Coats (2008).

As analysis of field-collected samples showed that *Epistylis* sp. attached mainly to adult *P. stuhlmanni*, experiments were conducted to determine whether *Epistylis* sp. would attach to copepodites in the absence of adults. Monocultures of copepodites (n = 35, ~500–800  $\mu\text{m}$  size) were set up as explained above and checked after 24 hours. Prevalence on these copepodites was then compared with prevalence on adult *P. stuhlmanni* (n = 25) also exposed to *Epistylis* sp. for 24 hours. To observe the effect of host density on epibiont prevalence, additional treatments with 5 and 40 *P. stuhlmanni* individuals were set up and checked after 24 hours. After the exposure time, the zooplankton were pipetted out of the petri-dishes with as little water as possible and into petri-dishes containing 50 mL of 5 % formalin, in order to ensure quick preservation and reduce the risk of detachment. The zooplankton were then immediately counted and examined for epibionts. Each monoculture and mixed culture petri-dish had three replicates and all experiments were repeated three times.

### ***Pseudodiaptomus stuhlmanni* survivorship**

*Pseudodiaptomus stuhlmanni* adults were sorted into three groups; A: non-hosting copepods; B: epibiont-hosting copepods with a low epibiont cover (< 20 % body covered by *Epistylis* sp.); and C: epibiont-hosting copepods with a high epibiont cover (> 40 % body covered by *Epistylis* sp.). Five *P. stuhlmanni* individuals corresponding to each group were placed in open 500 mL plastic jars filled with 250 mL of the 55- $\mu$ m filtered estuarine water.

*Pseudodiaptomus stuhlmanni* survives under laboratory conditions for ~ 5 weeks (pers. observ.). All experiments were conducted within 2 days of collection from the field, and the copepods were checked once per week over a 3-week period. On a daily basis, 50 mL of the 55  $\mu$ m filtered estuarine water was added into each plastic jar, to reduce the possibility of food deficiency and counter evaporative water loss. The medium was replaced with fresh medium on a weekly basis and in the process copepods were carefully sieved out onto petri-dishes and examined under the dissecting microscope (40  $\times$ ). The number of dead (un-responsive to mechanical stimuli), unhealthy (alive but with a low level of activity or with visible signs of physical injury) and healthy (active) copepods was recorded. Three replicate 500 mL jars per treatment were used and the experiment was repeated twice.

### **Statistical analysis**

Data were analysed on the R software platforms RStudio (version 3.2.1) and R Commander (version 2.1-7) for Windows. Because the epibiont prevalence data were categorical (epibiont-hosting and non-hosting individuals), Pearson's Chi-Square was used to test for statistical differences in the frequency of epibiont prevalence. Where the assumption of expected cell counts was violated, Fisher's Exact test was used. Where Chi-Square analysis of three treatments yielded significant p-values, the post-hoc analysis was conducted by determining the z-scores of each treatment. The p-values associated with these z-scores were then calculated and tested against a p-value of 0.01, which was attained after performing the Bonferroni correction to the p-value of 0.05.

A mixed-designs ANOVA was used to test for differences in the survivorship of group A: non-hosting *P. stuhlmanni*; group B: *P. stuhlmanni* with a low epibiont cover and group C: *P. stuhlmanni* with a high epibiont cover at the weekly intervals. The assumption of sphericity was met (Mauchly's test,  $p > 0.05$ ). Where significant differences were found, the Tukey HSD post-hoc test was used to determine the source of the differences.

## RESULTS

### *Epistylis* sp. host specificity and host epibiont density dependence

Except for the host-epibiont density dependence data, epibiont prevalence in the three runs of each experiment did not differ significantly ( $p > 0.05$  in all cases). Data for these experiments were therefore pooled for statistical analyses. The mixed culture experiments with the copepods *P. stuhlmanni*, *A. natalensis* and *O. brevicornis* showed that *Epistylis* sp. only attached to *P. stuhlmanni*, and this difference in epibiont prevalence was statistically significant ( $p < 0.01$ , Fisher's Exact test). The mixed culture experiments with *P. stuhlmanni* and the mysid *M. africana* produced a different result, as after 6 hours of exposure no epibionts were found attached to either species (Table 2.1).

The monoculture experiments revealed that *Epistylis* sp. did not attach to the other St Lucia zooplankton species even in the absence of *P. stuhlmanni*, as in these experiments only *P. stuhlmanni* harboured *Epistylis* sp. zooids after 6 hours of exposure ( $p < 0.01$ , Fisher's Exact Test; Table 2.2). Epibiont prevalence differed significantly between *P. stuhlmanni* adults and copepodites ( $p < 0.01$ ,  $\chi^2 = 75.1$ ), with lower prevalence in the copepodite treatments (Table 2.2). Assessment of the experimental petri-dishes showed no epibiont attachment to their surfaces.

Table 2.1: Epibiont prevalence on zooplankton mixed cultures comprising *O. brevicornis*, *A. natalensis* and *P. stuhlmanni* (A), or *M. africana* and *P. stuhlmanni* (B) exposed to *Epistylis* sp. for 6 hours.

Mixed culture	Species	n	Percentage prevalence (mean $\pm$ SD)		
			Experiment 1	Experiment 2	Experiment 3
A	<i>O. brevicornis</i>	36	0	0	0
	<i>A. natalensis</i>	36	0	0	0
	<i>P. stuhlmanni</i>	24	8.33 $\pm$ 14.4	12.5 $\pm$ 12.5	20.8 $\pm$ 7.22
B	<i>M. africana</i>	9	0	0	0
	<i>P. stuhlmanni</i>	30	0	0	0

Table 2.2: Epibiont prevalence on zooplankton monocultures exposed to *Epistylis* sp. for 6 and 24 hours.

Species	n	Percentage prevalence (mean $\pm$ SD)			Incubation period (hour)
		Experiment 1	Experiment 2	Experiment 3	
<i>O. brevicornis</i>	150	0	0	0	6
<i>A. natalensis</i>	150	0	0	0	6
<i>M. africana</i>	12	0	0	0	6
<i>P. stuhlmanni</i>	75	30.7 $\pm$ 16.2	28 $\pm$ 6.93	24 $\pm$ 10.6	6
<i>P. stuhlmanni</i>	75	28 $\pm$ 8	29.3 $\pm$ 16.2	34.7 $\pm$ 18	24
<i>P. stuhlmanni</i> copepodites	105	1.9 $\pm$ 1.65	0	0	24

### Host-epibiont density dependence

The first host-epibiont density dependence experiment revealed a significant difference in epibiont prevalence among the three densities of *P. stuhlmanni* ( $p < 0.05$ ,  $\chi^2 = 17.79$ ). Prevalence was lowest in the 5-individual treatment and highest in the 40-individual treatment. The z-scores and Bonferroni correction showed that these two treatments were responsible for the rejection of the null hypothesis, as they both had absolute z-scores higher than 1.96 (5-individual treatment:  $p < 0.01$ , z-score = 3.3; 25-individual treatment:  $p > 0.01$ , z-score = 1.9; 40-individual treatment:  $p < 0.01$ , zscore= -3.5). Epibiont prevalence in the three density treatments of the second and third experiments showed no significant difference ( $p > 0.05$ ,  $\chi^2 = 5.6$  [experiment 2],  $\chi^2 = 2.5$  [experiment 3]). Despite this, prevalence was higher in the 25 and 40 compared with the 5-individual treatment in all three experiments (Figure 2.1).

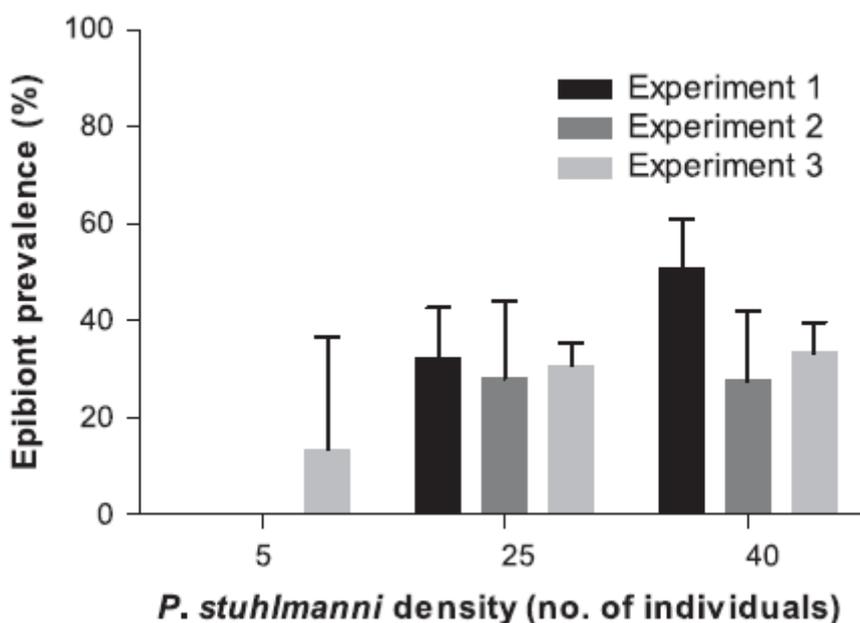


Figure 2.1: Prevalence of *Epistylis* sp. exposed to different densities of *P. stuhlmanni*. Zero-height bars represent treatments in which no epibiont attachment occurred. Error bars represent the standard deviation among replicate treatments in each experiment.

### Host survivorship

After 1 week of exposure in the first experiment, 87, 100 and 53 % survival was recorded in groups A (no epibionts), B (low epibiont cover) and C (high epibiont cover), respectively. After the second week, 80, 73 and 20 % survival was recorded in groups A, B and C, and after three weeks, 60, 53, and 13 %, respectively. In the second experiment, all copepods were alive in

groups A and B, while 33 % survival was recorded in group C after the first week of exposure. After the second week, 73, 87 and 0 % survival was recorded in groups A, B and C. After the third week, 47 and 40 % survival was recorded in groups A and B, respectively (Figure 2.2). In both experiments, live copepods from groups A and B were generally active, whereas those from group C displayed low motility. Inspection of the dead copepods from group C also revealed physical injury, as some of these copepods had broken antennae and urosomes. The mixed-designs ANOVA revealed a significant difference in survivorship among the three groups (experiment 1:  $p < 0.001$ ,  $F_{3,25,0.346} = 9.44$ ; experiment 2:  $p < 0.001$ ,  $F_{6,02,0.164} = 36.7$ ). A Tukey post-hoc test revealed that groups A and B did not differ from each other ( $p > 0.05$  in both experiments), but that both differed from group C ( $p < 0.001$  in both experiments). No interaction was observed between time and epibiont cover ( $p > 0.05$ ).

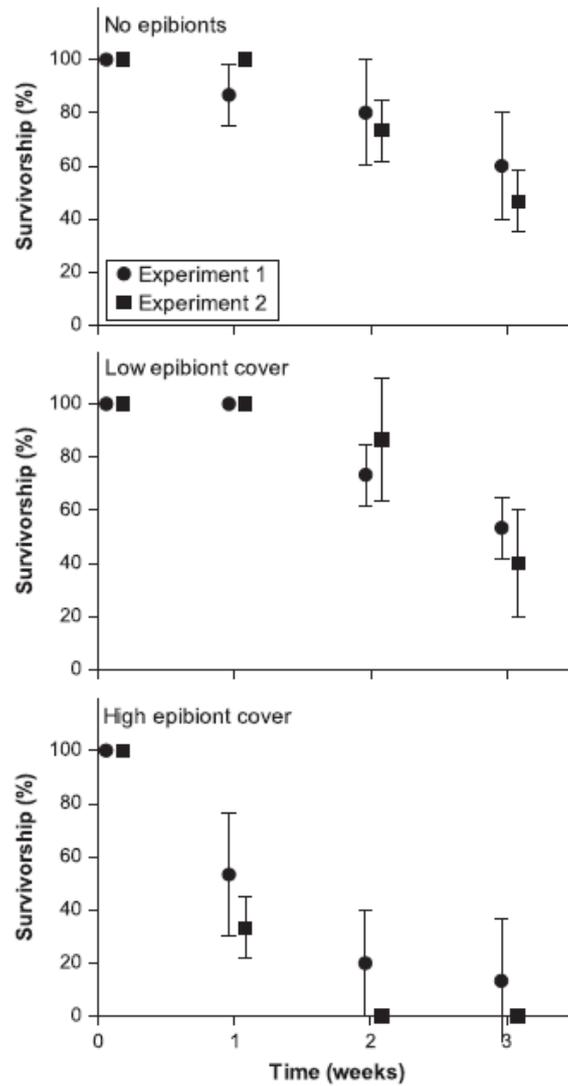


Figure 2.2: Survivorship of *P. stuhlmanni* with different percentage covers of the epibiont *Epistylis* sp. during a 3-week period. Error bars represent percentage standard deviation among replicate treatments in each experiment.

## DISCUSSION

This study has shown that the epibiotic peritrich *Epistylis* sp. selects specifically the calanoid copepod *P. stuhlmanni* and that high epibiont cover is associated with high copepod mortality rates in the St Lucia Estuary. Epibiont host specificity may be in part explained by the biological and ecological characteristics of the host (Willey et al. 1990; Chiavelli et al. 1993; Regali-Selegim and Godinho 2004). Compared with the other copepods investigated, *P. stuhlmanni* is relatively long-lived and larger in size and both factors might result in more encounters with the free-swimming stage of *Epistylis* sp.

In a study of zooplankton-ciliate epibiosis in the eutrophic Ashmore Lake, Illinois, Henebry and Ridgeway (1979) found a significant positive correlation between the size class of the cladoceran *Scapholeberis kingi* and cover by the peritrich *Vorticella microstoma*. Similarly, Regali-Selegim and Godinho (2004) investigated the prevalence of peritrich epibionts on the zooplankton of Monjolinho Reservoir in Brazil and found a higher prevalence on copepods than nauplii and rotifers. These authors suggested that the larger copepods were easier targets than the more numerically dominant, but physically smaller nauplii and rotifers.

The hypothesis of host-epibiont size dependence is, in the present study, supported by the exclusive attachment to *P. stuhlmanni* despite its lower numbers in the mixed culture copepod experiments and by the substantially higher prevalence on adult than copepodite *P. stuhlmanni*. However, the latter result, which has also been found in other studies, might also be attributable to the frequent moulting of the copepodite forms, which makes them a less stable substrate (Sherman and Schaner 1965; Henebry and Ridgeway 1979; Hanamura 2000). *Pseudodiaptomus stuhlmanni* is often the most abundant species in St Lucia and the host-epibiont density dependence experiments revealed a positive relationship between *P. stuhlmanni* abundance and prevalence of *Epistylis* sp.

Although host-epibiont density dependence in an epibiotic interaction may be taken to imply that the epibiont attaches to the most abundant species simply because of high encounter rates (Utz and Coats 2005 and references therein), it was clear from the monoculture experiments that *Epistylis* sp. does not attach to the other St Lucia species, even in the absence of *P. stuhlmanni*. This suggests that cues such as carapace texture and consistency might also be involved in locating and attaching to hosts (Wahl 2008). This hypothesis is consistent with the finding of a preliminary investigation, wherein *Epistylis* sp. was found to attach equally well

to *P. stuhlmanni* and the congeneric and morphologically similar *P. hessei*, collected from a different estuary in South Africa (Jones et al. unpublished data).

The mixed culture treatments with the mysid *M. africana* and *P. stuhlmanni* resulted in no attachment of *Epistylis* sp. to either species. Although not measured, the monoculture and mixed culture treatments with the mysid were observed to have fewer free-swimming *Epistylis* sp. than those with the other species at the end of the 6-hour incubation period. It is possible that the mysids release chemical cues that repel *Epistylis* sp. or hinder the ability of *Epistylis* sp. to sense *P. stuhlmanni*, thereby causing most of the *Epistylis* sp. zooids to remain attached to the dead host. However, the latter explanation is unlikely given that detachment occurred in the *A. natalensis* and *O. brevicornis* monocultures. In their motile form, epibionts are susceptible to predation by filter feeding zooplankton, including the species they attach to (Green 1974; Willey et al. 1990; Threlkeld and Willey 1993; Al-Dhaheri and Willey 1996; Holland and Hergenrader 1981; Barea-Arco et al. 2001). Being larger in size, *M. africana* is able to prey on the smaller zooplankton species (Carrasco and Perissinotto 2010). It is possible that this mysid, which has a higher clearance rate than the other species, exerted a high predation pressure on free-swimming *Epistylis* sp. zooids during the experiments, thereby preventing attachment to *P. stuhlmanni* and perhaps also to itself. To verify this, feeding experiments will have to be conducted in order to determine the predation pressure exerted by the mysid and the copepods on *Epistylis* sp.

By modulating the interaction of their host with other biotic as well as abiotic variables, epibionts may reduce the fitness of their hosts in several ways in the field (Wahl 2008). Willey et al. (1990) attributed the reduction in zooplankton hosting the epibiont *Colacium vesiculosum* to selective predation of the hosts by fish, and suggested that by increasing the apparent size of their host *C. vesiculosum* increased the susceptibility of their hosts to predation while also interfering with the escape responses. Epibionts may also compete with their zooplankton hosts for food in food-limited environments. Xu and Burns (1991) found that starving zooplankton hosts had a higher mortality rate than their non-hosting counterparts.

Epibiont cover may also be linked to copepod age, as older individuals may be weaker and therefore more susceptible to epibiont attachment (Weissman et al. 1993). Older individuals also have a longer time to accumulate epibionts than younger ones. Although similarly sized copepods were used in the survivorship experiments, it is possible that the highly infested

copepods were older and experienced higher mortalities because they were near the end of their life cycle. This could account for the higher mortality that was associated with high epibiont cover.

Despite its widespread occurrence, the ecological implications of epibiosis are still poorly understood, particularly at the ecosystem level (Wahl 2008). *Pseudodiaptomus stuhlmanni* plays an important role in the trophodynamics of St Lucia and adjacent freshwater systems, as it is a main food item of planktivorous and benthic fish species (Grindley 1982; Nhleko et al. 2012; Peer et al. 2013; Dyer et al. 2015). It is therefore important to better understand the impact of this copepod's interaction with *Epistylis* sp. Further research is needed to determine the physiological effect of *Epistylis* sp. on *P. stuhlmanni* and the environmental triggers of high *Epistylis* sp. densities, as well as to investigate how biotic factors such as predation pressure on *Epistylis* sp. may mediate the effects of this epibiont on *P. stuhlmanni* in St Lucia.

## **ACKNOWLEDGEMENTS**

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## Effects of the epibiont *Epistylis* sp. on the calanoid copepod host *Pseudodiaptomus stuhlmanni* assessed using the RNA:DNA ratio and RNA content in the St Lucia Estuary, South Africa

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### ABSTRACT

The calanoid copepod *Pseudodiaptomus stuhlmanni* from the St Lucia Estuary, South Africa is often host to the epibiotic peritrich ciliate *Epistylis* sp. Experiments have revealed low survivorship of heavily covered *P. stuhlmanni*. Studies showing the sub-organismal impact of epibiosis on zooplankton hosts are lacking. The RNA:DNA ratio and the RNA content are useful biochemical indicators of physiological well-being. In this study, these indicators were measured in epibiont-hosting and non-hosting *P. stuhlmanni* individuals. The RNA:DNA ratio of hosting copepods was lower ( $0.187 \pm 0.036$ ) than that of non-hosting copepods ( $0.234 \pm 0.105$ ) however, this difference was not significant ( $U = 25$ ;  $p > 0.05$ ). The RNA content of hosting *P. stuhlmanni* was significantly lower than that of non-hosting copepods (hosting copepods =  $134 \pm 41.2$  ng. copepod<sup>-1</sup>; non-hosting copepods =  $170 \pm 41$  ng. copepod<sup>-1</sup>;  $t = -2.49$ ,  $df = 31$ ,  $p < 0.05$ ). These findings contribute new information on the physiological impacts of epibiosis however, the RNA values obtained for both hosts and non-hosts are lower than most reported for similar taxa. Therefore, I recommend conducting more nucleotide extractions with other copepods in St Lucia and in other estuaries in this sub-tropical region.

**Keywords:** fitness, nutritional state, peritrich, physiology

### INTRODUCTION

Copepods are the most abundant metazoans in aquatic environments (Turner 2004; Guo et al. 2012) and serve a central role in the trophic transfer of energy from primary producers to organisms occupying higher trophic levels (Williams et al. 1994; Speekmann et al. 2007). Copepods are often hosts of epibiotic ciliates, which attach to the exoskeleton of these organisms. Herman and Mihursky (1964) and Utz (2003) recorded the ciliate *Zoothamnium* sp. attached to the calanoid copepod *Acartia tonsa* in Chesapeake Bay, USA. Burris and Dam (2014) also recorded this epibiotic association in Long Island Sound, USA. Utz (2003) and Souissi et al. (2013) recorded this epibiont on *Eurytemora affinis* in Chesapeake Bay and the Seine Estuary in France, respectively.

The peritrich ciliate *Epistylis* sp. has been documented on calanoid copepods such as *A. tonsa* (Utz 2003 in Chesapeake Bay), and *A. bifilosa* (Visse 2007, Gulf of Riga in the Baltic Sea), as well as on the cyclopoid copepod *Mesocyclops* sp. from Lake Velachery, India (Rajabunizal and Ramanibai 2011). Several studies have shown that epibiotic ciliates can reduce the fitness of their copepod hosts, by decreasing mating success (Souissi et al. 2013), egg production rate (Weissman et al. 1993) and longevity (Visse 2007; Jones et al. 2016b [Chapter 2]). Given the abundance and role of copepods in aquatic food webs, epibionts have the potential to affect the carbon influx of aquatic environments.

Limited studies have attempted to show the sub-organismal mechanisms through which epibionts affect host fitness (Hakimzadeh and Bradley 1990; Petkeviciute et al. 2015). Such assessments are important and they may reveal sub-lethal impacts that may not be detected through short-term fitness experiments. Biochemical indices, such as the RNA:DNA ratio and RNA content are useful in determining the potential secondary production and nutritional state of organisms (Wagner et al. 1998; Speckmann et al. 2007). Protein is the main body compound in most organisms and ribosomal RNA (rRNA) is an essential precursor of protein synthesis (Ikeda et al. 2007; Gorokhova et al. 2014).

A high RNA concentration in body tissue reflects mainly rRNA and consequently, a high potential for protein synthesis (Wagner et al. 1998; Speckmann et al. 2007). In contrast, a low RNA concentration may be found when organisms are subjected to stress (Wagner et al. 2001), indicating that energy is allocated to stress responses rather than protein synthesis growth and reproduction. DNA content generally remains constant in mature organisms irrespective of stress levels and, therefore, acts as an index of cell number (Bulow 1987; Buckley et al. 1984). The RNA:DNA ratio and RNA content may therefore be used to assess the nutritional condition and growth rate of zooplankton (Båmstedt and Skjoldal 1980; Wagner et al. 1998; Buckley et al. 1999; Becker et al. 2005; Yebra et al. 2017). The RNA:DNA ratio has been successfully applied in comparisons of starved and well fed cladocerans (Vrede et al. 2002), as an indicator of copepods in diapause (Wagner et al. 1998) and to test the effects of UV radiation on copepods (Lagos et al. 2015). RNA content has been used to test the effects of temperature on copepods (Wagner et al. 2001) and as a tool to determine zooplankton growth rates (Båmstedt and Skjoldal 1980; Saiz et al. 1998).

*Epistylis* sp. is a peritrich ciliate that attaches to *Pseudodiaptomus stuhlmanni*, a calanoid copepod in the St Lucia Estuary, South Africa (Figure 1.1). Experiments have revealed that while low epibiont cover (< 20 % host body covered by epibionts) has no effect on the survivorship of *P. stuhlmanni*, longevity is reduced at high cover (> 40 % host body covered by epibionts; Jones et al. 2016b [Chapter 2]). In this study, the RNA:DNA ratio and RNA content were used to test the physiological effect of *Epistylis* sp. on *P. stuhlmanni*.

## MATERIALS AND METHODS

### Preliminary extractions

Given the relatively small size of *P. stuhlmanni* adults (~ 1 mm), it was necessary to pool individuals in order to get an accurate quantification of RNA and DNA. Males and females were also pooled, in order to have an adequate number of replicates. To determine the optimal number of individuals to pool, a preliminary analysis was conducted. A zooplankton sample was collected from Dredger Harbour within St Lucia (Figure 1.1) in March 2017, using a hyperbenthic sled fitted with a 100 µm mesh net. Thereafter, a glass micropipette was used to isolate adult *P. stuhlmanni* individuals with no attached epibionts under a Kyowa SDZ dissecting microscope (40 ×). Copepods were sorted into three replicate batches of 10, 20, 40, 60 and 80 and immediately placed in a -80 °C freezer until nucleic acid extraction. TRIzol reagent (Invitrogen, Cat. 15596026), which maintains the integrity of RNA (see Hummon et al. 2007) was used to isolate the RNA and DNA.

To isolate RNA from DNA and proteins, the TRIzol reagent (500 µL) was pipetted into Eppendorf tubes, with each tube containing a batch of copepods. The samples were then homogenized (Qiagen Tissuelyzer, 50 Hz, 1 min), incubated (5 min), mixed with chloroform (100 µL), incubated (3 min) and centrifuged (15 min at 12 000 × g). Three distinct layers formed, with a clear aqueous layer at the top of the tube which contained RNA, an interphase containing DNA and a bottom red layer with proteins. After the aqueous RNA layer was carefully pipetted into a new tube, the DNA and proteins were refrigerated (4 °C).

The RNA was then precipitated through the addition of isopropanol (250 µL), incubation (10 min) and centrifugation (20 min at 12 000 × g). To remove any contaminants (DNA, proteins or phenol), the RNA pellet was washed by centrifugation (5 min at 7500 × g) while immersed in ethanol (500 µL of 75 %). After discarding the supernatant, the pellet was air-dried (~ 8 min), solubilized in RNase-free water (50 µL) and placed on a heat block (15 min, 55 °C). A

Nanodrop Spectrophotometer ND-1000 was thereafter used to quantify RNA. Absorbance was measured at 260 nm and 280 nm for RNA quantification and assessment of purity (A<sub>260</sub>/A<sub>280</sub>).

To precipitate DNA, 300 µL ethanol (100 %) was pipetted into each DNA and protein-containing tube, mixed by inversion of the tube, incubated (3 min) and thereafter centrifuged (5 min at 2 000 × g). The DNA pellet was isolated from the supernatant, which contained proteins. To wash the DNA (i.e. remove any remaining RNA, protein or phenol), the pellet was suspended in sodium citrate (500 µL), incubated (30 min) and centrifuged (5 min at 2000 × g). After discarding the supernatant, the sodium citrate, incubation and centrifugation process was repeated once or twice, depending on pellet size.

The DNA pellet was then suspended in 75 % ethanol (1 mL), incubated (15 min) and centrifuged (5 min at 2 000 × g). After discarding the supernatant, the pellet was air-dried (~ 8 min). The pellet was solubilized by suspension in sodium hydroxide (8 mM, 300 µL) and centrifugation (10 min at 12 000 × g). The Nanodrop Spectrophotometer ND-1000 was then used again to quantify DNA at the absorbance described above. Each RNA and DNA concentration was corrected for dilution in the final re-suspension step (50 and 300 µL respectively).

After the RNA and DNA values of the different batches were obtained, they were plotted against batch size and regression analyses were conducted in order to determine the linearity of the relationship between nucleotide yield and copepod batch size. Copepod batch size accounted for 92 % of the variability in RNA ( $R^2 = 0.917$ ) while for DNA it accounted for 93 % of the variability ( $R^2 = 0.93$ ; Figure 3.1). One-way analysis of variance (ANOVA) was conducted to check if there was any difference in the RNA:DNA ratio obtained from the different batches. No difference was found ( $F_{4,10} = 1.72$ ,  $df = 4$ ,  $p > 0.05$ ), meaning that with an increase in batch size, the RNA and DNA values experienced the same unit increase. The pellet size of nucleic acids from the small batches was difficult to see, and this likely caused the variability in the RNA:DNA ratio of the batches comprising 10 individuals (Figure 3.2). Therefore, batches comprising at least 20 individuals were used for all subsequent extractions.

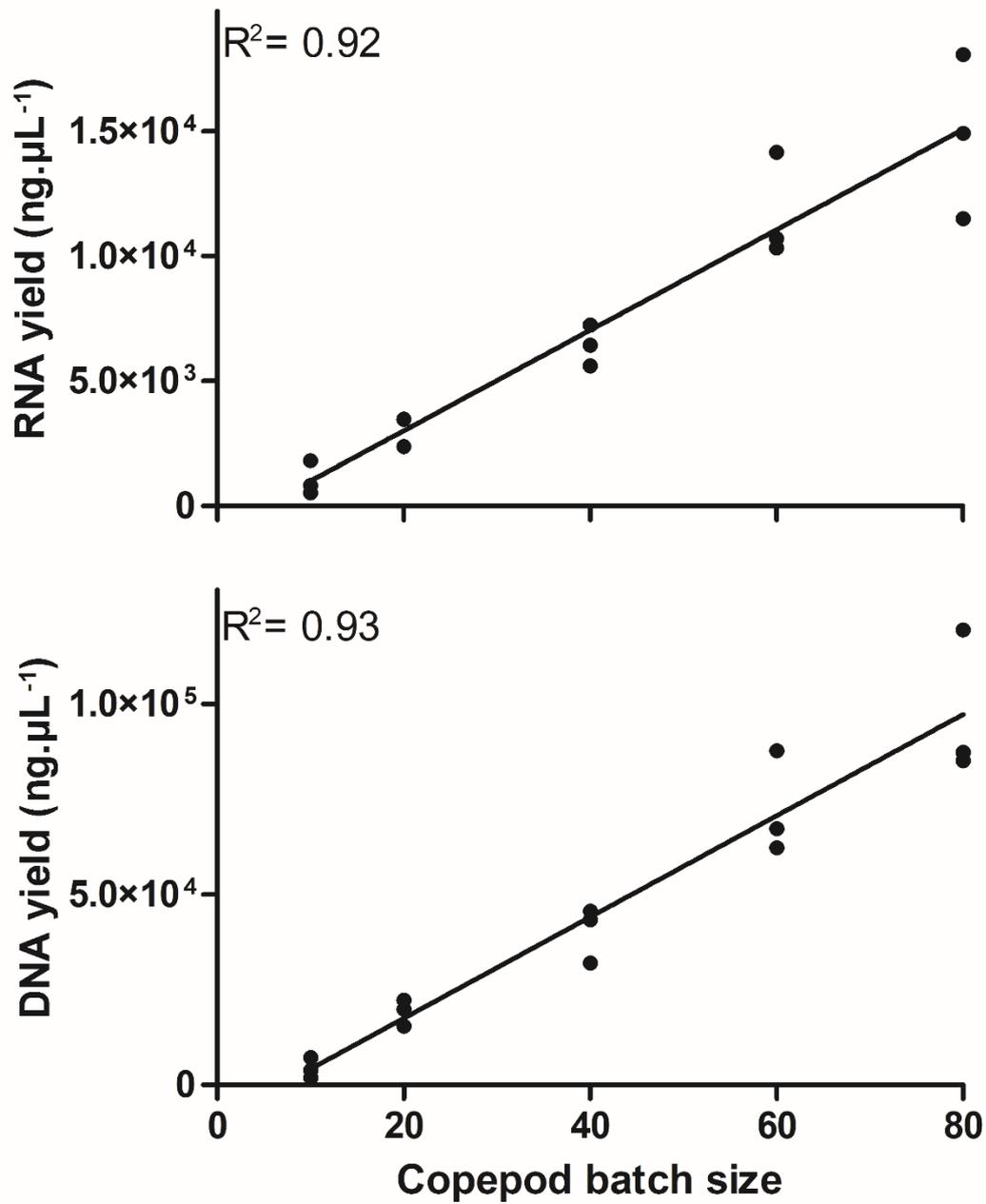


Figure 3.1: RNA and DNA yield (ng.µL<sup>-1</sup>) of different batch sizes of *Pseudodiaptomus stuhlmanni*.

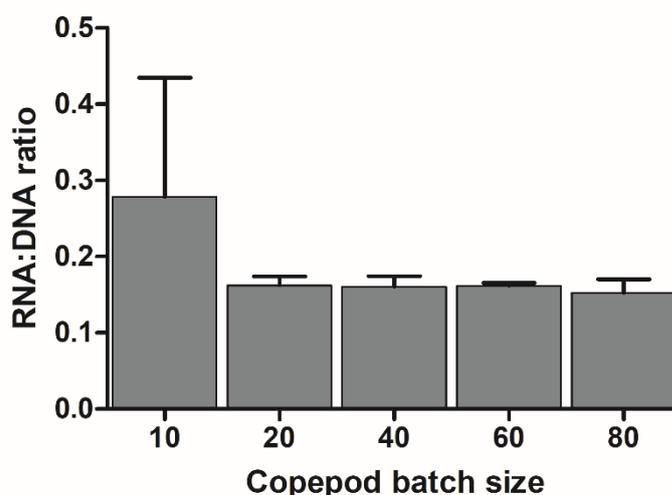


Figure 3.2: RNA:DNA ratio of different batch sizes of *Pseudodiaptomus stuhlmanni*. Error bars represent standard deviation.

#### **RNA:DNA ratio of epibiont-hosting and non-hosting *Pseudodiaptomus stuhlmanni***

After the preliminary extractions, more copepods were collected from Dredger Harbour in May and October 2017 for subsequent extractions to determine the RNA:DNA ratio of epibiont-hosting and non-hosting copepods. Due to the low number of heavily covered copepods recovered from St Lucia in 2017, only copepods with a low (< 20 %) to medium (20–40 %) *Epistylis* sp. cover were used. To avoid quantification of the peritrichs' nucleic acids, epibionts had to be removed from the hosting copepods. Before being frozen (-80 °C), live epibiont-hosting copepods were killed by crushing their cephalosomes with fine-tipped forceps, and thereafter manually removing all attached zooids and stalks as quickly as possible.

To remove any ciliate zooids and other contaminants, each copepod was rinsed in distilled water before being placed on ice with other dead individuals during this process. Thereafter, each batch was immediately frozen (-80 °C) until nucleic acid extraction (as outlined above). The treatment of the epibiont-hosting copepods might have affected their RNA content and to account for this, the copepods with no attached epibionts were treated in the same manner before also being frozen. In total, 1080 copepods from Dredger Harbour were used for RNA:DNA ratio analysis. From these, 560 individuals hosted epibionts and these were divided among 7 batches. The remaining 420 were non-hosts and were divided among 9 batches.

#### **RNA content of epibiont-hosting and non-hosting *Pseudodiaptomus stuhlmanni***

In order to determine if the RNA content would corroborate the findings of the RNA:DNA ratio and could be used as a biochemical indicator in future studies, the RNA content of non-hosting and epibiont-hosting copepods was compared. In addition to the RNA content of the 1080 copepods from Dredger Harbour, RNA was also extracted from copepods collected at another site in St Lucia, Charter's Creek. A total of 1485 copepods from Charter's Creek (collected in October 2017) were isolated. From these, 720 were epibiont-hosting and divided among 9 batches. The remaining 765 copepods were non-hosts and divided among 8 batches. Therefore, in total RNA was extracted from 33 copepod batches (16 hosts, 17 non-hosts). After correcting the nucleotide values for dilution as stated above, concentration was also corrected for the number of copepods in each batch, so that nucleotide content is expressed in ng. copepod<sup>-1</sup>.

### Statistical analyses

Simple linear regression, ANOVA, student's t-tests and the Mann-Whitney U test were conducted in this study. The assumptions were met for all but one dataset (RNA:DNA ratio, assumption of homoscedasticity violated). For this dataset, the non-parametric Mann-Whitney U was conducted. All statistical analyses were conducted on IBM SPSS v.25 for Windows. The results are reported as mean  $\pm$  standard deviation.

## RESULTS

The purity levels of the nucleic acids were  $A_{260}/A_{280} = 1.91 \pm 0.212$  for RNA and  $A_{260}/A_{280} = 1.3 \pm 0.176$  for DNA. There was no significant difference in the RNA content of copepods collected at the two sites (Dredger Harbour and Charter's Creek) and on the different dates (May and October 2017; One-way ANOVA,  $F_{2,30} = 0.012$ ,  $df = 2$ ,  $p > 0.05$ ). Therefore, these data were pooled before statistical comparisons of the RNA content of hosting and non-hosting copepods. The average values were  $170 \pm 41$  ng. copepod<sup>-1</sup> for non-hosts and  $134 \pm 41$  ng. copepod<sup>-1</sup> for hosts (Figure 3.3). This difference in RNA content was significant ( $t = -2.49$ ,  $df = 31$ ,  $p < 0.05$ ).

The DNA content of non-hosts was  $770 \pm 270$  ng. copepod<sup>-1</sup> whereas that of hosting copepods was  $785 \pm 143$  ng. copepod<sup>-1</sup> (Figure 3.3). There was no significant difference in the DNA content of the two groups (t-test,  $t = 0.126$ ,  $df = 14$ ,  $p > 0.05$ ). The RNA:DNA ratio of non-hosts ranged from 0.1 to 0.37 and that of hosts ranged from 0.146 to 0.231 (Figure 3.3).

Although the mean RNA:DNA ratio of non-hosting copepods was higher ( $0.234 \pm 0.105$ ) than that of hosts ( $0.187 \pm 0.036$ ), this difference was not significant ( $U = 25$ ,  $p > 0.05$ ).

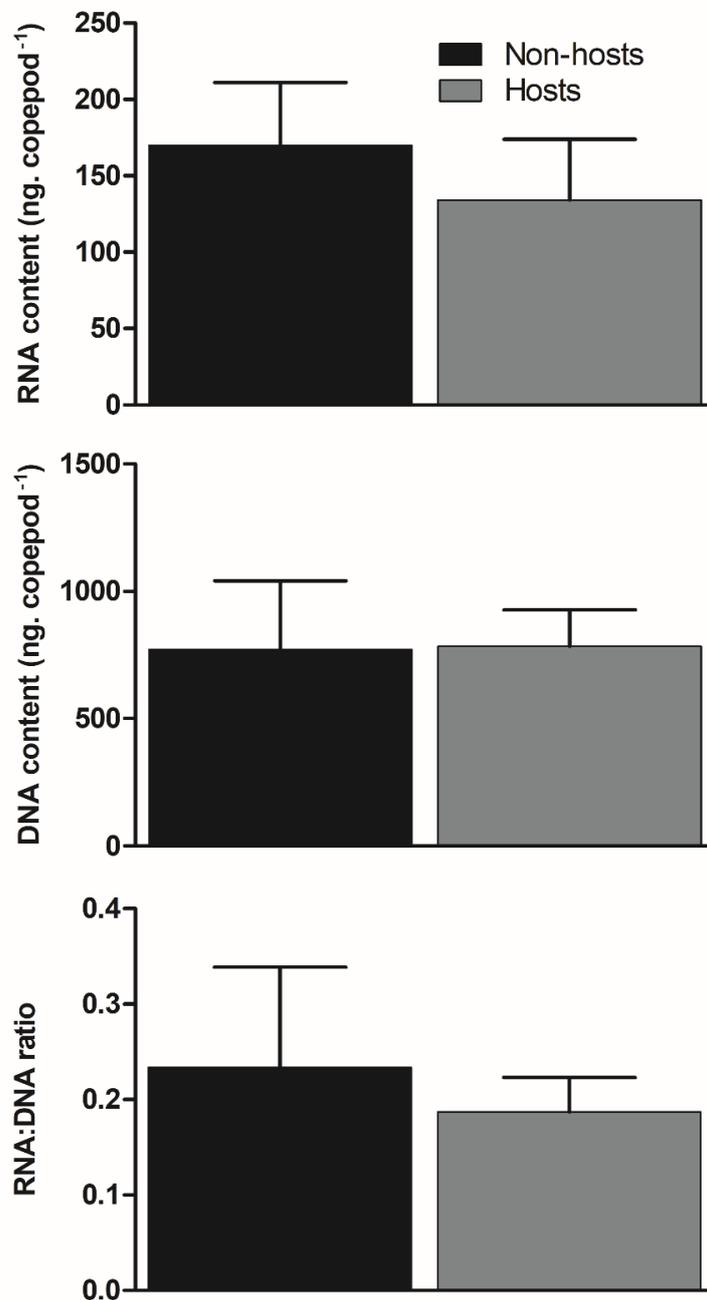


Figure 3.3: RNA and DNA content (ng. copepod<sup>-1</sup>) as well as the RNA:DNA ratio of epibiont-hosting and non-hosting *Pseudodiaptomus stuhlmanni*. Error bars represent standard deviation.

## DISCUSSION

The aim of this study was to determine the effects of the epibiotic peritrich *Epistylis* sp. on the physiological well-being of the host copepod *P. stuhlmanni*, through assessments of the RNA:DNA ratio and RNA content of this calanoid. Although the difference was not significant, the RNA:DNA ratio of epibiont-hosting *P. stuhlmanni* was lower than that of non-hosts. The RNA content of hosting copepods was significantly lower than that of non-hosts.

There are several ways in which epibionts can reduce the energetic status and consequently the RNA content, of their hosts. Epibionts are a physical burden (Allen et al. 1993; Carman and Dobbs 1997) and this can be energetically costly if the hosts have to divert resources meant for processes such as growth and reproduction, towards supporting the epibiont load. Souissi et al. (2013) used video recordings to capture and compare the trajectory of the calanoid *Eurytemora affinis* hosting the peritrich *Zoothamnium* sp. to that of non-hosting individuals. These authors found that hosting males were less active, exhibiting fewer jumps and taking longer breaks between movements than non-hosting males.

Souissi et al. (2013) also found both epibiont-hosting males and females to have a lower mean speed and an increased sinking frequency. This suggests that the infested copepods lacked the energy to maintain their position in the water column. In contrast, Weissman et al. (1993) found slower sinking rates for the calanoid *Acartia tonsa* hosting *Epistylis* sp. These authors suggested that the larger surface area created by *Epistylis* sp. increased drag. Increased drag would make it more energetically costly for copepods to migrate downward and in this way affect the host's ability to find food and mates, as well as escape predators.

After investigating the effects of *Zoothamnium* sp. on various fitness components of the host *A. tonsa*, Burris and Dam (2014) demonstrated that *Zoothamnium* sp. decreased the fitness of this copepod. Among their findings was that hosting copepods were physically smaller than their non-hosting counterparts, which also points to decreased investment in growth. The specific site of attachment on the host body may play a role in dictating the physiological response (Souissi et al. 2013 and references therein). Epibiont attachment to legs mechanically hinders locomotion and therefore impedes feeding. Attachment to antennae and mouth parts physically interferes with ingestion and epibionts may intercept food particles in the host's feeding current. In the long term, a decrease in food quantity or quality can result in depletion of energy reserves.

The attachment of *Epistylis* sp. via stalk erection on the exoskeleton of its hosts may also play a role in reducing RNA content. SEM images revealed surface damage and bacteria around the area where *Epistylis* sp. stalks had been erected on *P. stuhlmanni* (see Chapter 1, Figures 1.6 A and B). This is similar to observations by Turner et al. (1979) who found bacteria around lesions made by *Epistylis* sp. colonising *A. tonsa* in Escambia Bay, Florida. Turner et al. (1979) proposed this bacterial infection and loss of copepod body fluid as a factor contributing to the seasonal succession of copepod species in estuaries.

The findings of the survivorship experiments detailed in Jones et al. (2016b [Chapter 2]) show that *Epistylis* sp. reduced the longevity of heavily covered *P. stuhlmanni* individuals. On the other hand, the survivorship of *P. stuhlmanni* individuals with a low epibiont cover was higher and not statistically different to that of copepods with no attached epibionts. As mainly copepods with a low to medium (up to 40 %) body cover were found in field-collected samples during this study, the effects of heavy infestation on the RNA:DNA ratio and RNA content of *P. stuhlmanni* could not be tested. The findings of this study can therefore be interpreted to mean that *Epistylis* sp. does not have to attain high cover to affect the health of *P. stuhlmanni* in the long term.

Very few studies have investigated the physiological response of zooplankton to epibiont infestation. Through the use of [<sup>35</sup>S] methionine-labelling and autoradiography, Hakimzadeh and Bradley (1990) were able to detect up-regulation of heat shock proteins in *E. affinis* hosting unidentified algal epibionts. Petkeviciute et al. (2015) investigated the response of *A. tonsa* to infestation by the epibiont *Colacium vesiculosum* by assessing expression of stress-related genes. While these authors found no response of heat shock proteins (Hsp70 and Hsp90) to infestation, they found significant up-regulation of ferritin in hosting *A. tonsa* individuals. Ferritin over-expression has been linked to bacterial infections (Petkeviciute et al. 2015 and references therein) and oxidative stress (Orino et al. 2001) and Petkeviciute et al (2015) interpreted the up-regulation in epibiont-hosting *A. tonsa* as a physiological response induced by *C. vesiculosum*.

The current study contributes to investigations of the physiological impacts of epibiosis and is the first to link effects at the individual level (Jones et al. 2016b [Chapter 2]), with sub-organismal impacts. The findings also have implications at the ecosystem level. In St Lucia as well as in surrounding rivers, *P. stuhlmanni* is a key prey item for higher trophic level taxa

(Grindley 1982; Nhleko et al. 2012; Peer et al. 2013; Dyer et al. 2015). Therefore, irrespective of what the ramifications of low RNA content and RNA:DNA ratio are at the individual and population level, there are food web implications as predators consuming even moderately infested *P. stuhlmanni* get less nutrition per unit effort.

During this study, over 1600 adult copepods were used for determination of the RNA:DNA ratio (including the preliminary extractions) and in spite of some variability in the data, the ratio ranged from 0.1 to 0.4, consistently lower than those reported for other calanoids. The ratios most comparable to those found in this study were reported by Wagner et al. (1998), who investigated the suitability of the RNA:DNA ratio as an indicator of the nutritional state of the calanoid *Calanus finmarchicus*. Wagner et al. (1998) found ratios ranging from 0.46 to 1.48 in low food treatments and from 1.05 to 3.37 in high food treatments.

In contrast, Speekmann et al. (2007) reported ratios up to 10 and 14 for starved and well-fed *A. tonsa*, respectively. Ikeda et al. (2007) found ratios ranging between 0.86 and 2.65 for calanoids collected from different depths, with the lowest ratio recorded for the deep-sea species. These authors also found that carnivores had lower ratios than detritivores and suspension feeders, and that stage C6 females had higher ratios than C6 males and other stages. Gorokhova et al. (2007) and Pommier et al. (2010) reported ratios of ~3.5 and 10.3 respectively for different populations of *E. affinis*. Consequently, RNA:DNA ratio appears to be species and/or population-specific, and should be investigated and interpreted with care. At St Lucia, the RNA content of copepods collected from the different sites (Dredger Harbour and Charter's Creek) was not significantly different and this shows that within this system the impact *Epistylis* sp. has on the nutritional state of *P. stuhlmanni* is not site-specific.

In summary, the findings of this study show that *P. stuhlmanni* individuals hosting *Epistylis* sp. have a lower nutritive value than their non-hosting counterparts. This corroborates the negative effects on fitness found in Jones et al (2016b [Chapter 2]). Because the findings of this study support the consistency of DNA content in *P. stuhlmanni*, rather than resorting to laborious experimentation, the RNA:DNA ratio and/or RNA content can be used in any future studies aimed at inferring potential fitness effects in *P. stuhlmanni*.

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## Turbidity effects on the attachment success of the peritrich epibiont *Epistylis* sp. in the St Lucia Estuary, South Africa

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### ABSTRACT

The peritrich *Epistylis* sp. is often observed attached to the dominant copepod *Pseudodiaptomus stuhlmanni* during turbid conditions in the St Lucia estuarine lake, South Africa. This study aimed to test the effects of turbidity (comprising both natural and inorganic silt) on the attachment success of *Epistylis* sp. *Epistylis* sp. was isolated and exposed to potential *P. stuhlmanni* hosts for 24 hours under six turbidity treatments across the range 8–1500 NTU. The prevalence and density of *Epistylis* sp. exposed to inorganic silt decreased significantly across the turbidity range in both runs of this experiment. In the natural silt treatments, epibiont prevalence increased with turbidity up to 500 and 250 NTU in the first and second experiment, respectively. Beyond these peaks prevalence decreased, although non-linearly in the second experiment. Density peaked at 250 NTU in both experiments and generally followed a similar trend to that of prevalence. The increase in epibiont prevalence and density in the natural silt experiments is directly related to the high organic matter content in this treatment. Association of *Epistylis* sp. with turbidity may impact negatively on *P. stuhlmanni*, as the longevity of this copepod is negatively related to heavy cover by *Epistylis* sp. and to turbidity.

**Keywords:** ciliate, copepod host, nutrition, *Pseudodiaptomus stuhlmanni*, suspended silt

### INTRODUCTION

Ciliate epibionts are common components of aquatic ecosystems, where they are found attached to a diverse range of metazoans (Henebry and Ridgeway 1979; Green and Shiel 2000; Fernandez-Leborans 2010). These organisms may benefit by feeding on the host's excreta and in the host's feeding current, hiding from predators and dispersing at little energetic cost (Barea-Arco et al. 2001; Fernandez-Leborans 2010). The hosts may also possibly benefit by being camouflaged from predators (Stoecker 1978). However, most studies have shown that ciliate epibionts generally have a negative impact on zooplankton hosts (e.g. Visse 2007;

Gilbert and Schröder 2003; Souissi et al. 2013). Epibionts can be metabolically costly as they impose a physical burden on zooplankton (Xu and Burns 1991; Allen et al. 1993; Threlkeld and Willey 1993; Chapter 3). They may also attach to unsuitable areas such as on the cephalosome and on swimming appendages and can therefore interfere with vital processes including food acquisition, swimming and mating. Further, rather than camouflage, epibionts may make their host physically conspicuous to visual predators and compete with their hosts for nutrients and food if the size range of food overlaps (Kankaala and Eloranta 1987; Wahl 1989; Xu and Burns 1991).

Epibiont presence may be quantified in various ways, including as density (total number of attached epibiont zooids per host) and prevalence (percentage of host population hosting epibionts) and is influenced by several factors. Biotic determinants of ciliate epibionts include predation pressure, prey and host availability as well as competition (Henebry and Ridgeway 1979 and references therein; Wahl 1989) while environmental drivers may include salinity, temperature and pollution (Green 1974; Fernandez-Leborans 2010; Souissi et al. 2013). Early studies into the ecology of epibiotic ciliates revealed an association with pollution and eutrophic conditions (Henebry and Ridgeway 1979 and references therein). This may be a result of increased food availability or increased susceptibility of host populations to epibiont attachment during sub-optimal conditions.

*Epistylis* sp. is a colonial, stalked peritrich ciliate and is commonly found attached to *Pseudodiaptomus stuhlmanni*, a dominant calanoid copepod in the St Lucia estuarine lake situated in KwaZulu-Natal, South Africa. St Lucia is Africa's largest estuarine lake and plays a vital role as a nursery for estuarine-dependent taxa in the region (Whitfield and Taylor 2009). St Lucia is prone to turbid conditions caused by both the inflow of sediment-laden freshwater and sediment re-suspension during windy conditions (Whitfield and Taylor 2009; Zikhali et al. 2015). Turbidity has a generally negative effect on biota as photosynthesis may be reduced due to light attenuation, sediment particles may be abrasive and gut contents may be diluted by ingestion of the non-nutritious sediment. By affecting some species more than others, turbidity may also play a structuring role in ecosystems and affect the outcome of predator-prey relationships, competition and symbiosis (Arruda et al. 1983; McCabe and O'Brien 1983; Anthony and Fabricius 2000).

Studies conducted on three dominant St Lucia zooplankton species, namely the mysid *Mesopodopsis africana* and the calanoid copepods *Acartiella natalensis* and *P. stuhlmanni*, showed that they are all negatively affected by turbidity, with both feeding and survivorship being compromised (Carrasco et al. 2007, 2013; Jones et al. 2015). However, *P. stuhlmanni* exhibited a higher tolerance, surviving for longer at the highest turbidity treatments than the other two species (Jones et al. 2015). *Pseudodiaptomus stuhlmanni* has also been found surviving under a wide salinity and turbidity range in the field (Grindley 1982; Jones et al. 2016a). Given that *Epistylis* sp. compromises the fitness of *P. stuhlmanni* (Chapters 2 and 3), it is possible that *Epistylis* sp. decreases the tolerance of this copepod to sub-optimal conditions. On the other hand, turbidity might protect *P. stuhlmanni* from infestation if *Epistylis* sp. is less tolerant of turbidity. This study aims to test the effects of turbidity on the attachment success of *Epistylis* sp. to *P. stuhlmanni* in the St Lucia Estuary.

## **MATERIALS AND METHODS**

### **Sample collection**

The calanoid copepod *P. stuhlmanni* and attached *Epistylis* sp. were collected at Charter's Creek and the Mouth in the St Lucia Estuary with a D-net mounted on a hyperbenthic sled and fitted with a 100 µm mesh (Figure 1.1). The sled was towed in the near-shore zone for 30 m and the retained organisms were transported back to the laboratory in buckets along with estuarine water and sediment. The collections and experiments were conducted in July and December 2015, during the regional dry and wet season respectively.

### **Experimental design**

To isolate the effects of turbidity due to silt in combination with organic matter from those of silt alone, parallel experiments were conducted, one using natural silt and one using inorganic silt. Sediment collected at the St Lucia Estuary was sieved through a 63 µm mesh in order to trap silt-sized particles. This silt and 55 µm filtered (to exclude larger grazers) estuarine water were used to set up different turbidity levels. Although turbidity levels as high as 2500 Nephelometric Turbidity Units (NTU) have been recorded in St Lucia's main freshwater source, the adjacent Mfolozi River, values higher than 1500 NTU are seldom reached within St Lucia itself. Therefore, the turbidity treatments chosen for this study were 50, 250, 500, 1000 and 1500 NTU, and were attained by adding silt to the 55 µm filtered estuarine water. The control was ~ 8 NTU. A HACH 2100Qis turbidimeter was used to measure turbidity.

To attain inorganic silt, St Lucia sediment was combusted for 8 hours in a muffle furnace (420 °C). After cooling, it was placed in 2 L buckets with 0.2 µm filtered, autoclaved estuarine water, in order to soften it. The sediment was thereafter sieved through a 63 µm mesh net to attain silt-sized particles. This silt and the 55 µm filtered estuarine water were then used to attain the turbidity levels as described above.

In order to determine the amount of food available per turbidity treatment, microalgal biomass (measured as the sum of chlorophyll a and phaeopigments) was determined for each turbidity treatment and experiment type (turbidity comprised of natural and inorganic silt). To determine the microalgal biomass and its size class composition, duplicate sets of 80 mL of each turbidity treatment were size-fractionated by being sequentially filtered through 20, 2 and 0.7 µm filters. The size-fractionation was conducted in order to determine the amount of algae available to *Epistylis* sp. for consumption, as this epibiont has an average peristomial lip width of 20 µm (Chapter 1) and can therefore only feed on organic matter smaller than 20 µm. The filters were placed in test tubes containing 6 mL of 90 % acetone for 48 hours of cold extraction of pigments. The microalgal biomass in each sample was thereafter measured fluorometrically using a Turner Designs 10-AU fluorometer.

Formation of free-swimming telotroch stages of epibiotic peritrichs may occur due to reproduction, host moulting or host mortality (Clamp 1973; Utz and Coats 2008). These telotrochs then begin searching for a host substrate. To test the effects of turbidity due to natural silt on the attachment success of *Epistylis* sp., infested copepods were killed by transferring them into a glass slide with a drop of water and piercing the cephalosome with fine-tipped forceps under a Kyowa SDZ dissecting microscope (40 ×). The dead copepods were immediately pipetted into petri-dishes containing 50 mL of the 55 µm filtered estuarine water.

The *P. stuhlmanni* individuals from samples collected in July hosted a higher density of epibionts compared with individuals collected in December. Therefore the number of copepods used to isolate telotrochs was lower (46 individuals) in the July experiments than the December experiments (between 80 and 95 individuals). The dead copepods were left in petri-dishes up to four hours, in order to attain a telotroch density of ~ 10 telotrochs per mL. The copepod carcasses were thereafter removed from the petri-dishes, the remaining telotrochs were emptied into 500 mL honey jars and the volume of 55 µm filtered estuarine water adjusted to the 350 mL mark. Therefore, there were about 500 telotrochs per 350 mL of water. Uniform

distribution of telotrochs was ensured by closing the honey jar and gently inverting it, before 10 mL of the water (with ~ 14 telotrochs) was pipetted out of the honey jars into 100 mL bottles containing 80 mL water corresponding to each turbidity level. All turbidity treatments were set up to account for the 10 mL dilution, so that they were all within 5 NTU of their desired level. Ten adult, non-hosting *P. stuhlmanni* individuals were then pipetted into each bottle.

There were five 100 mL bottles for each turbidity level and this experiment was repeated twice. The same procedure was followed to test the effects of turbidity due to inorganic silt on *Epistylis* sp. As both experiments (natural and inorganic silt) were conducted at the same time on each occasion, the same control (~ 8 NTU) treatment was used. All bottles were placed on a laterally-rotating (3 rpm) plankton wheel. This was done to mimic sediment resuspension in situ and maintain the turbidity levels. After 24 hours, the bottles were removed from the wheel and the copepods gently sieved out, into petri-dishes with the 55 µm filtered estuarine water and analysed for attached *Epistylis* sp. zooids under the dissecting microscope. Epibiont prevalence and density were recorded.

### **Statistical analyses**

Data were analysed on the R software platform RStudio (version 3.2.1) for Windows. The relationship between microalgal biomass and turbidity was tested with correlation analysis. To do this, the microalgae data from the different size classes was combined. All but one dataset met the assumptions of Pearson's correlation. As the dataset corresponding to the second inorganic silt experiment violated the assumption of normality, Spearman's Rank correlation was performed on this dataset.

The relationship between microalgal biomass and epibiont attachment (prevalence and density) was tested with regression analyses. A quadratic regression was conducted on the data corresponding to natural silt experiments, as these data violated the assumption of linearity. Linear regression was conducted on the data corresponding to the inorganic silt experiments.

To test for significant turbidity effects on epibiont prevalence and density, One-way ANOVA was carried out on data corresponding to each experiment. The assumptions of normality and equal variance were tested on studentized residuals using the Shapiro-Wilk test and the Levene's test, respectively. Assumptions were met for the natural silt experiments and post hoc comparisons were performed using the Tukey test in order to determine the source of statistical differences. However, the assumptions of normality and equal variance were not met for the

inorganic silt experiments, even after data transformation (arcsine and log for prevalence and density data, respectively). Therefore, the non-parametric Kruskal-Wallis test was performed and assumptions for this test were met. The Dunn's test with Bonferroni adjustment was thereafter conducted in order to check the source of any differences.

## **RESULTS**

### **Relationship between turbidity and organic matter**

Correlation analysis showed a significant and positive relationship between turbidity and microalgal biomass in both the natural silt experiments (experiment 1:  $r = 0.952$ ,  $p < 0.001$ ; experiment 2:  $r = 0.986$ ,  $p < 0.001$ ; Figure 4.1 A). The relationship between turbidity and microalgae was also significant but negative in the inorganic silt experiments (experiment 1:  $r = -0.877$ ,  $p < 0.001$ ; experiment 2:  $r_s = -0.989$ ,  $p < 0.001$  Figure 4.1 B). The dominant microalgal size class across the turbidity gradient was nanoplankton, followed by microplankton for both the natural and inorganic silt experiments (Figure 4.2).

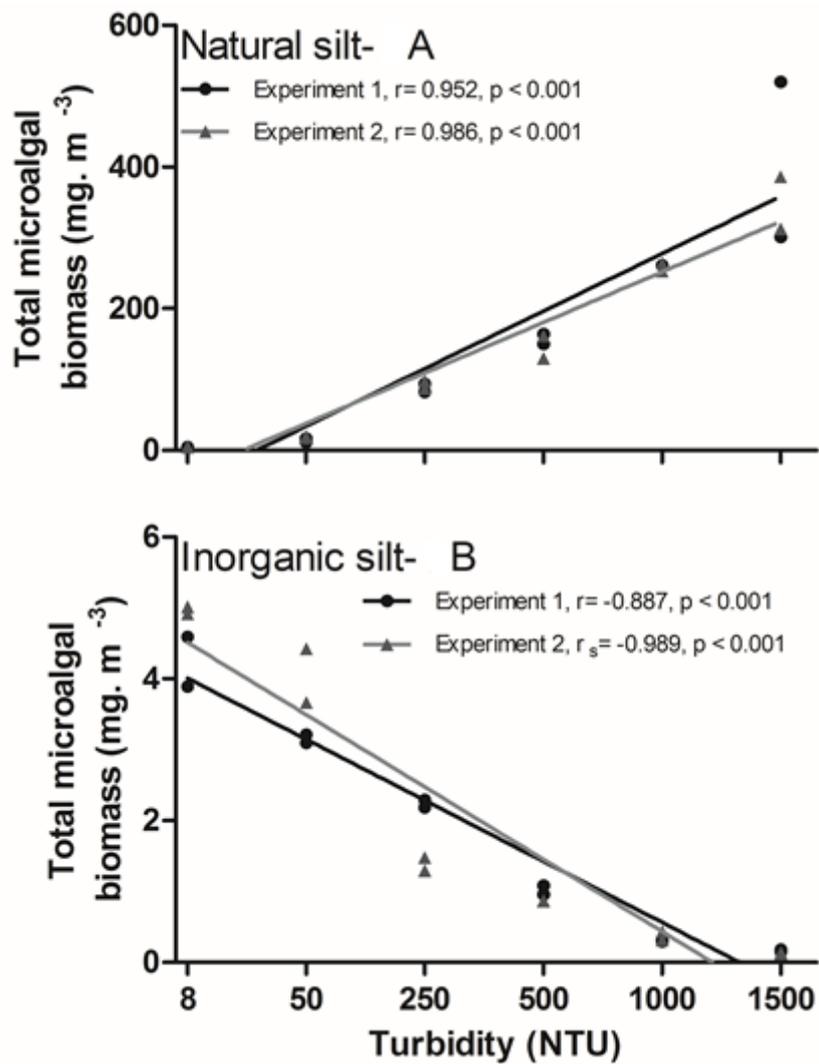


Figure 4.1: Relationship between total microalgae and turbidity. The  $r$  values represent Pearson's correlation coefficient and the  $r_s$  value represents Spearman's rank correlation coefficient ( $n = 2$  measurements). Note the different scales on the y-axes.

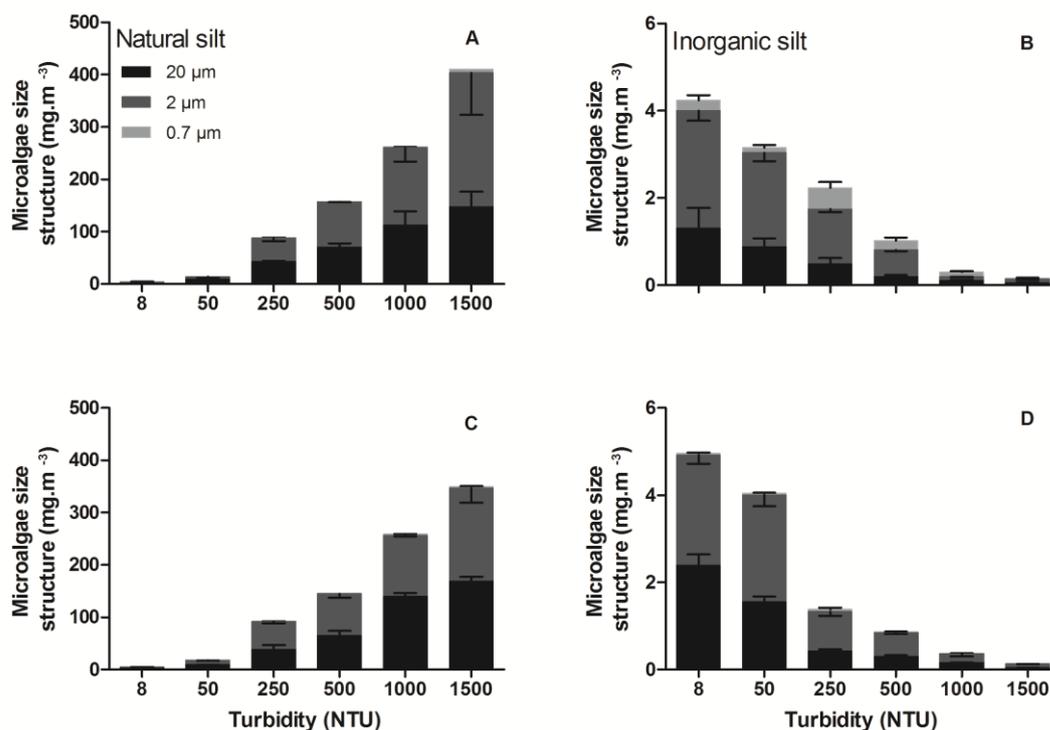


Figure 4.2: Size-fractionated microalgae biomass corresponding to each of the six turbidity treatments (natural silt experiments: A and C; inorganic silt experiments: B and D). Bars and error bars respectively represent mean and standard error of  $n=2$  replicate measurements. Note the different scales on the y-axes.

## Turbidity effects on epibiont prevalence and density

### *Natural silt experiments*

Turbidity due to natural silt significantly affected epibiont prevalence (ANOVA, experiment 1:  $F_{1957,162} = 12.1$ ,  $df = 5$ ,  $p < 0.001$ ; experiment 2:  $F_{1501,245} = 6.13$ ,  $df = 5$ ,  $p < 0.001$ ). For the first experiment, the Tukey post hoc test revealed that the control treatment (8 NTU) did not significantly differ from 50 and 1500 NTU but significantly differed from the other treatments. The 50 and 1500 NTU treatments each differed from 500 and 1000 NTU. Overall the two lowest (8 and 50 NTU) and the highest (1500 NTU) treatments had the most similar and lowest prevalence, as prevalence increased from the control until 500 NTU and thereafter decreased to produce a bell-shaped curve (Figure 4.3 A).

In the second experiment, the trend in prevalence in the lower turbidity range was similar to that found in the first, with an increase from 8 to 250 NTU. However, there was no clear trend across the 250–1500 NTU range and apart from 500 NTU, prevalence was generally variable

within each of these treatments (Figure 4.3 A). The Tukey post hoc test revealed a significant difference between 8 NTU and 250, 500 and 1500 NTU as well as between 50 and 250 NTU. All other treatments did not significantly differ from each other.

In both experiments, the highest *Epistylis* sp. density was found in 250 NTU and the lowest in the control (Figure 4.3 C). Epibiont density was significantly affected by turbidity (ANOVA, experiment 1:  $F_{47.3,9.93} = 4.76$ ,  $df = 5$ ,  $p < 0.001$ ; experiment 2:  $F_{57.8,13.2} = 4.38$ ,  $df = 5$ ,  $p < 0.001$ ). In the first experiment, epibiont density in the 8 NTU treatment differed from that in 250, 500 and 1000 NTU. There were no other differences among treatments. The significant turbidity effect in the second experiment is only attributable to a difference between 8 and 250 NTU, as no other treatments differed significantly.

### ***Inorganic silt experiments***

The trend in both experiments was a decrease in prevalence across the 8–1500 NTU range, with the control and 50 NTU treatment having the highest prevalence in both experiments. There were no attached epibionts across the 500–1500 NTU (experiment 1) and the 1000–1500 NTU (experiment 2) treatments (Figure 4.3 B). The Kruskal-Wallis tests revealed a significant turbidity effect on epibiont prevalence (experiment 1:  $\chi^2 = 22.3$ ,  $df = 5$ ,  $p < 0.001$ ; experiment 2:  $\chi^2 = 13.6$ ,  $df = 5$ ,  $p < 0.05$ ). For experiment 1, Dunn's test showed that the control treatment (8 NTU) differed from the three highest treatments (500–1500 NTU;  $p < 0.01$  in all cases). No other treatments differed from each other. Significant differences in the second test were between the control (8 NTU) and the two highest treatments (1000 and 1500 NTU;  $p < 0.05$  in both cases).

The highest epibiont density was recorded at 8 NTU in both experiments. In the first experiment, the lowest density was across the 500–1500 NTU range, as no attached epibionts were observed. In addition, no epibionts were observed in the 1000 and 1500 NTU treatments in the second experiment (Figure 4.3 D). Turbidity significantly affected epibiont density (Kruskal-Wallis, experiment 1:  $\chi^2 = 23.4$ ,  $df = 5$ ,  $p < 0.001$ ; experiment 2:  $\chi^2 = 14.6$ ,  $df = 5$ ,  $p < 0.05$ ). In the first experiment, 8 NTU differed from all treatments except 50 NTU, while in the second experiment 8 NTU differed from 1000 and 1500 NTU treatments. No other differences in density were observed among treatments in either experiment.

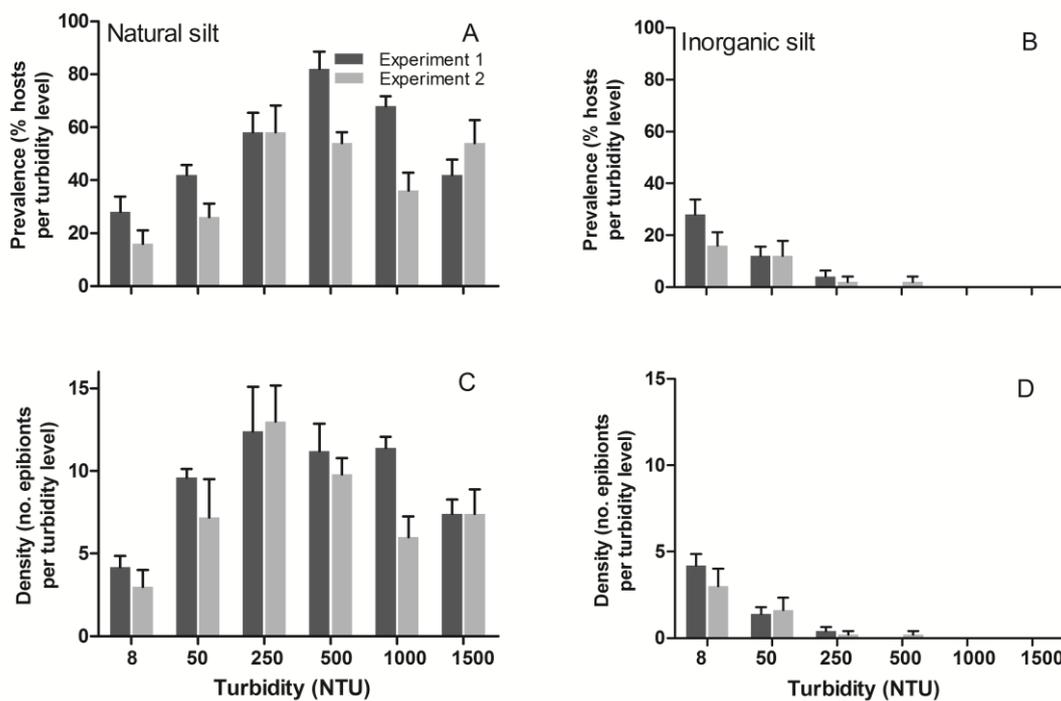


Figure 4.3: Prevalence and density of the epibiont *Epistylis* sp. exposed to the copepod *P. stuhlmanni* in a range of turbidities comprised of natural (A and C) as well as inorganic (B and D) silt for 24 hours. Zero-height bars represent lack of attachment; bars and error bars respectively represent mean and standard error of  $n = 5$  replicate measurements. Note the different scales on the y-axes.

#### Organic matter effects on epibiont prevalence and density

Microalgal biomass was positively related to epibiont presence. Regression analysis revealed significant effects for all but one experiment (density versus microalgae, natural silt experiment 2). The strength of the relationships was variable, ranging from  $R^2 = 0.639$  to  $R^2 = 0.118$  (Table 4.1).

Table 4.1: Regression analyses of the relationship between organic matter content and epibiont prevalence and density. The quadratic and linear regression provided the best fit for the natural and inorganic silt experiments, respectively. Degrees of freedom= 27 and 28 for the quadratic and linear regression, respectively.

			Experiment 1 Microalgae	Experiment 2 Microalgae
Natural silt (quadratic regression)	Prevalence	R <sup>2</sup>	0.623	0.251
		p	< 0.001	< 0.05
	Density	R <sup>2</sup>	0.269	0.118
		p	< 0.05	> 0.05
Inorganic silt (linear regression)	Prevalence	R <sup>2</sup>	0.611	0.44
		p	< 0.001	< 0.001
	Density	R <sup>2</sup>	0.639	0.477
		p	< 0.001	< 0.001

## DISCUSSION

The aim of this study was to determine the relationship between the attachment success of the epibiotic peritrich *Epistylis* sp. and turbidity in the St Lucia estuarine lake. Turbidity made up of inorganic silt resulted in a decrease in prevalence and density of this epibiont across the 8–1500 NTU turbidity gradient. Because the inorganic silt had no attached bio-film of organic material, it was more abrasive and of no nutritional value. These particles may have interfered with the feeding of the epibiont and host by blocking phytoplankton and through gut loading of the silt. The abrasive particles could also directly harm the cells. These effects of the inorganic silt may have resulted in premature mortalities of the epibionts. On the other hand, turbidity made up of natural silt mixed with organic matter content resulted in a higher epibiont prevalence and density, although the trend was non-linear and inconsistent between experiments at the highest turbidities (500–1500 NTU). Microalgal biomass was positively related to epibiont presence, meaning that it was the organic matter associated with the silt particles rather than the silt that caused the increase in prevalence and density with an increase in organically rich turbidity.

Peritrichs generally feed on bacteria and nanoplankton cells (Utz and Coats 2005 and references therein). As the dominant phytoplankton size class in this study was nanoplankton, the high organic matter content in the natural silt treatments may have influenced reproduction through a positive effect on the epibionts' feeding rate. Several studies have indicated that epibionts are able to intercept their host's feeding current and may also use nutrients derived

from the host's excreta depending on their site of attachment (Holland and Hergenrader 1981; Barea-Arco et al. 2001 and references therein). The organically rich turbidity may also indirectly benefit *Epistylis* sp., if this epibiont derives supplementary nutrition from the feeding current and excreta of *P. stuhlmanni*, as this copepod has a high feeding rate in turbidity treatments with natural silt (Jones et al. 2015).

The increase in *Epistylis* sp. prevalence from the lowest treatment to 500 NTU (experiment 1) and to 250 NTU (experiment 2) and the increase in density up to 250 NTU (both experiments) in the natural silt treatments suggests that organically rich turbidity is beneficial to *Epistylis* sp., but only up to a certain threshold. Therefore, the findings of this study suggest that during turbid conditions in the St Lucia Estuary, suspended solids may represent either a stress factor or a source of nutrition for *Epistylis* sp. and that this is governed by the quantity as well as the organic matter content of the suspended solids. St Lucia is shallow (depth < 2 m) and wind action readily suspends fine particles (Zikhali et al. 2015). As the main freshwater source for St Lucia is the degraded Mfolozi River, riverine inflow is also a significant contributor of suspended silt (Carrasco et al. 2007; Whitfield and Taylor 2009; Nhleko et al. 2012). Carrasco et al. (2007) recorded turbidities up to 2588 NTU in 2006 at the Mfolozi Mouth. Nhleko et al. (2012) recorded turbidities > 1000 NTU in 2009 at the Mfolozi Estuary and its mouth. Following a flood event in March 2014, the Narrows region had turbidity of 962 and 308 in March and April 2014, respectively (Jones et al. 2016a).

The findings of this study also have implications for the population dynamics of the host species *P. stuhlmanni*. The survivorship of this copepod is negatively affected by high turbidity (Jones et al. 2015) and by high *Epistylis* sp. cover (Jones et al. 2016b [Chapter 2]). Any synergistic effect of epibiosis and turbidity might result in a decline in the abundance of the *P. stuhlmanni* population in St Lucia. As this species often numerically dominates the zooplankton community (Carrasco et al. 2010; Carrasco and Perissinotto 2015) and plays a key role in linking lower level taxa with macrofauna and fish in St Lucia (Grindley 1982; Nhleko et al. 2012; Peer et al. 2013; Dyer et al. 2015), the trophic transfer of energy may also be negatively affected.

In conclusion, this study has shown that the presence of *Epistylis* sp. is positively related to nutritious turbidity but negatively related to inorganic turbidity. St Lucia has an ongoing problem of silt loading and regularly experiences high turbidities. More studies are needed in

order to have a better understanding of the implications for the *P. stuhlmanni* population and the food webs of the entire ecosystem. In order to understand the ecology of *Epistylis* sp., future studies also need to consider the relationship between this peritrich epibiont and other potential drivers, such as salinity and temperature.

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## Effects of salinity, temperature and turbidity on the survivorship of the epibiotic peritrich *Epistylis* sp. in the St Lucia Estuary, South Africa

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### ABSTRACT

Although epibiosis is a widespread form of symbiosis, studies that experimentally show the environmental drivers of this relationship are lacking. The peritrich ciliate *Epistylis* sp. is commonly found attached to the dominant calanoid copepod *Pseudodiaptomus stuhlmanni* in the St Lucia Estuary, South Africa. A study was conducted to test if salinity interacted with temperature and turbidity to affect the survivorship of *Epistylis* sp. Two-Way Analysis of Variance on Ranks was conducted to test for the main and interaction effects of these parameters. There was no interaction between temperature and salinity, and temperature did not affect *Epistylis* sp. The survivorship of *Epistylis* sp. was however significantly and negatively affected by high salinity. Post hoc analyses revealed that the highest salinity level (25) was responsible for most of the mortalities. The survivorship of *Epistylis* sp. was significantly affected by the interaction between turbidity and salinity. High turbidity (1000 NTU) negatively affected survivorship across the entire salinity gradient used (0–25), and high salinity (25) also generally resulted in low survivorship across the turbidity gradient of 50–1000 NTU. As the host, *P. stuhlmanni*, is more tolerant of high salinity and high turbidity than *Epistylis* sp., these findings suggest that these sub-optimal conditions can modulate epibiosis by limiting the prevalence and cover of *Epistylis* sp. in the St Lucia Estuary.

**Keywords:** ciliate epibiont, copepod host, physico-chemical drivers

### INTRODUCTION

Peritrich ciliates are abundant in the aquatic environment and are often found attached to crustacean hosts in a symbiotic association known as epibiosis (Xie et al. 2001; Fernandez-Leborans 2010). Due to the dynamic nature of aquatic environments, at any given time the prevailing physico-chemical conditions may be within the range tolerated by the hosts but perhaps not by the epibionts, and vice versa (Wahl 1989; Threlkeld et al. 1993). Therefore, although the occurrence of epibionts is necessarily dependent on host availability,

environmental factors modulate epibiosis. However, experimental evidence of the environmental drivers of epibiosis is scarce (Fernandez-Leborans 2010).

In estuarine environments, salinity and temperature are considered to be the dominant drivers of the distribution of biota (Dorgelo 1976; Kinne 1971; Velasco et al. 2006). Temperature affects physiological parameters such as metabolism and growth rate and may operate in concert with salinity and other factors to influence the occurrence of biota (Dorgelo 1976). Salinity dictates species distribution along the freshwater–ocean continuum. Salinity stress may result in re-allocation of energy that would have otherwise been used for somatic growth and reproduction, towards osmoregulation (Beyrend-Dur et al. 2011 and references therein). Because the aquatic environment is dynamic, the fluctuation of physico-chemical factors also manifests in the temporally variable distribution of taxa within the same water body.

*Epistylis* sp. is a peritrich ciliate commonly found attached to *Pseudodiaptomus stuhlmanni*, a dominant calanoid copepod in the St Lucia estuarine lake located in the subtropics of South Africa. The St Lucia Estuary undergoes cyclic wet and dry phases (Begg 1978) and during the dry phases, the system experiences high salinities, with hypersaline conditions establishing in the northern lake basins. Because of agricultural activities, water from the adjoining Mfolozi River carries with it a high silt load, which is discharged into St Lucia following heavy rainfall. Therefore, resident St Lucia biota have to contend with a wide range of fluctuating parameters, including salinity, temperature and turbidity. Studies in St Lucia have shown that turbidity negatively affects the dominant zooplankton, by hindering feeding and/or respiration and by reducing longevity (Carrasco et al. 2007, 2013; Jones et al. 2015). Other negative effects of high turbidity on biota include limitation of photosynthetic activity, physical harm to soft-bodied organisms and dilution of gut contents (Dejen et al. 2004).

The nature of the epibiotic relationship can be detrimental for the host (de Pádua et al. 2013; Bielecka and Boehnke 2014; Pane et al. 2014) and high *Epistylis* sp. cover (percentage of host individual covered by epibionts) has a negative association with the survivorship of *P. stuhlmanni* (Jones et al. 2016b [Chapter 2]). This copepod is euryhaline (Grindley 1976, 1982) and is comparatively more tolerant of high turbidity than the other dominant zooplankton species found at St Lucia (Jones et al. 2015 and references therein). *Epistylis* sp. may however reduce the tolerance of this copepod to sub-optimal environmental conditions. Conversely, conditions sub-optimal to this epibiont may relieve the host *P. stuhlmanni* from heavy

epibiont infestation. It is therefore important to better understand the relationship between the attached stage of *Epistylis* sp. and abiotic variables. The aim of this study is to determine how the interaction of: a) salinity with temperature; and b) salinity with turbidity affects the survivorship of *Epistylis* sp.

## **MATERIALS AND METHODS**

### **Sample collection**

Zooplankton were collected using a hyperbenthic sled fitted with a D-net with a mesh size of 100  $\mu\text{m}$  at Charter's Creek and the Narrows in the St Lucia Estuary (Figure 1.1). The collections and experiments were conducted between December 2015 and December 2016.

### **Acclimation to salinity treatments**

The acclimation was conducted because a preliminary shock exposure experiment revealed a high mortality rate for ciliates taken from the field at a salinity of  $\sim 8$  and placed directly into medium with test salinities of 15 and above. This mortality was reduced when ciliates were acclimated stepwise in increments between 2 and 5 every 2 to 3 hours. Increasing the acclimation time beyond 3 hours (up to 8 hours) did not affect mortality. The different salinity treatments were set up by filtering dam water through a 0.7 $\mu\text{m}$  GF/F filter, then adding "Instant Marine" aquarium salts to the water. The treatments were prepared at discrete salinity levels of 2.5, 5, 10, 15, 20, 25, 30 and 35, with pure dam water (with a salinity of 0.3) serving as the fresh treatment. Low salinities are typically recorded in St Lucia after heavy rainfall and/or freshwater inflow and high salinity levels are recorded after prolonged periods of freshwater deprivation or after saltwater intrusion. The treatments were set up 24 hours before the commencement of the experiments to ensure that the aquarium salt had dissolved, and salinity was checked with the ATAGO S/Mill-E hand refractometer.

Epibiont-hosting *P. stuhlmanni* individuals collected in the field (salinity, temperature and turbidity  $\sim 10$ , 24  $^{\circ}\text{C}$  and 81 NTU) were isolated under a Kyowa SDZ dissecting microscope (40 $\times$ ) in a controlled temperature (21 $^{\circ}\text{C}$ ) room. The copepods were then placed in 500 mL glass jars containing 90 mL of the salinity medium with cultured *Chlorophyta* algae (Supplementary material 5.1) at a concentration of  $\sim 4$  mg pigm. $\text{m}^{-3}$ , which is within the range of phytoplankton recorded in the Narrows and Charter's Creek during routine sampling. The concentration was verified with a Turner Designs 10-AU non-acidification fluorometer. For target salinity treatments  $< 8$ , the copepods were first incubated at a salinity of five. After 3

hours, ~ 65 % of the copepods were transferred into the 2.5 salinity treatment and after another 3 hours, half of the copepods in the 2.5 treatment were placed in pure dam water. The same process was performed at the higher salinities, up to 35. The ciliates were left to acclimatize in their final treatments for ~3 hours before 15 epibiont-hosting copepod individuals were isolated from each salinity treatment and inspected under the dissecting microscope (40 ×).

High epibiont mortality occurred in the salinity treatments across the range 25–35, even after the stepwise acclimation and acclimation for > 3 hours did not improve survivorship. Epibiont mortality (measured as detachment or desiccation/inactivity) occurred on nine out of the 15 copepods sub-sampled from the salinity treatment of 25 and no epibionts survived the 30–35 salinity range. Therefore, the range 0–25 was used in all subsequent experiments, which were all conducted after acclimation. Although mortality occurred during the acclimation process before each experiment, only that recorded during the experiments is reported in this study.

#### **Salinity-temperature effects on *Epistylis* sp. survivorship**

Following acclimation, one epibiont-hosting copepod was placed into an 11 mL vial corresponding to its test salinity and then into a water bath set at one of three temperatures: 15, 21 or 30 °C. These temperatures are within the range typically recorded at St Lucia and were verified with a mercury thermometer. Each salinity-temperature treatment was filtered through a 0.7 µm mesh, aerated and contained a *Chlorophyta* concentration of ~4 mg pigm.m<sup>-3</sup>. Each combination was replicated ten times (ten vials) and these vials were incubated for 24 hours in the water baths, to make up 21 temperature-salinity combinations. These experiments were conducted twice, in December 2015 (field salinity, temperature and turbidity ~8, 29 °C and 64 NTU) and February 2016 (field salinity, temperature and turbidity ~2, 19 °C and 211 NTU).

#### **Salinity-turbidity effect on *Epistylis* sp. survivorship**

The turbidity treatments used were 50, 250 and 1000 NTU, which are within the normal range measured in St Lucia (Carrasco et al. 2007). To make up the different turbidity treatments, sediment was collected in the St Lucia Estuary and sieved through a 63 µm mesh in order to trap silt-sized particles. This silt was then added to 2 L buckets with water corresponding to each of the salinity treatments (0–25) and stirred in order to have a homogenous mixture. While the silt particles were in suspension, a subsample was taken and turbidity was measured using a HACH 2100Qis turbidimeter. As the silt was mixed with organic matter or had organic matter adhered to it, the *Chlorophyta* culture was not used as a food source for these experiments.

Each 11 mL vial was filled with 9 mL of one of the salinity-turbidity treatments and one copepod. Each salinity-turbidity combination was replicated ten times and placed on a rotating plankton wheel (3 rpm) to keep the silt particles in suspension. The incubation was conducted for 24 hours at 21 °C in a controlled temperature room and under night conditions. Thereafter, the copepods were carefully sieved out into petri-dishes and examined under the dissecting microscope. These experiments were conducted twice, in June (field salinity, temperature and turbidity ~0.5, 26 °C and 316 NTU) and December 2016 (field salinity, temperature and turbidity ~2, 28 °C and 139 NTU). In all experiments, only copepods with one epibiont colony were used and each colony had between 6 and ~ 14 zooids. Mortality was only recorded if all zooids in a colony were inactive or if all zooids had detached.

### **Statistical analyses**

Data were analysed with the software Statistica version 13 for Windows. Because the data were ranked (0 = no attached/live epibionts, 1 = live epibionts) Two-way Analysis of Variance (ANOVA) on Ranks (Conover and Iman 1981) was conducted in order to test for the main effects and interaction effects.

## **RESULTS**

### **Salinity-temperature effect on *Epistylis* sp. survivorship**

There was no interaction between salinity and temperature (Experiment 1:  $F_{12,189} = 0.718$ ,  $p > 0.05$ ; Experiment 2:  $F_{12,189} = 1.01$ ,  $p > 0.05$ ). Temperature had no effect on epibiont survivorship (Experiment 1:  $F_{2,189} = 1.44$ ,  $p > 0.05$ ; Experiment 2:  $F_{2,189} = 0.875$ ,  $p > 0.05$ ). In contrast, salinity significantly affected epibiont survivorship (Experiment 1:  $F_{6,189} = 8.24$ ,  $p < 0.001$ ; Experiment 2:  $F_{6,189} = 8.93$ ,  $p < 0.001$ ). In both runs of the experiment, the highest salinity level (25) had the lowest survivorship. A Tukey post hoc test conducted on the Experiment 1 data revealed that survivorship at the salinity of 25 was significantly different from that observed at salinities of 0, 5 and 15. No other treatments differed. The post hoc conducted on the Experiment 2 data showed that survivorship in treatments across the gradient 0–20 was significantly different from survivorship at 25, but not from each other. Survivorship was generally high across the salinity range 0–15 and the fluctuations within this range had no distinct trend. Survivorship was lowest at 25 (Figure 5.1).

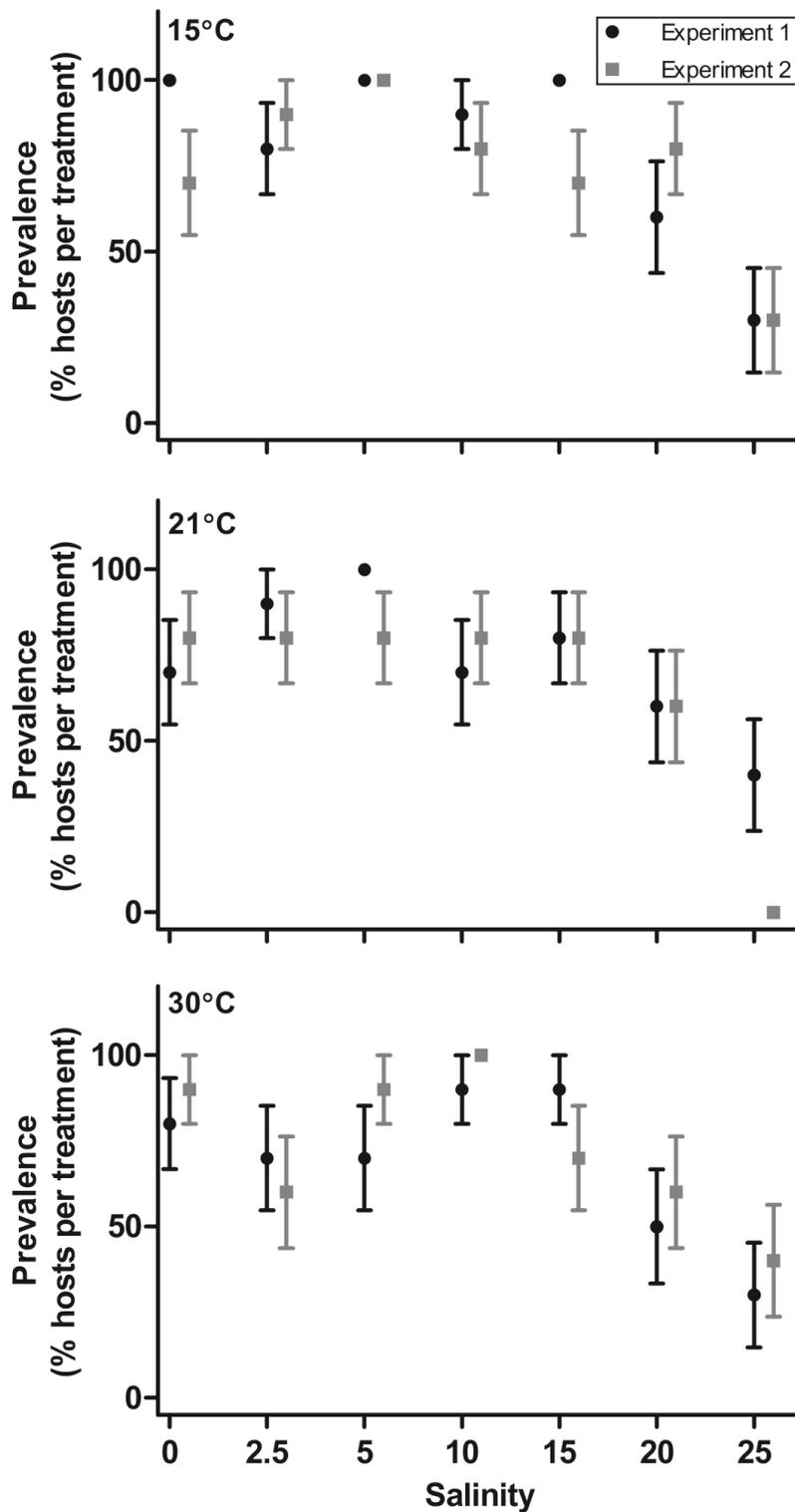


Figure 5.1: Survivorship of the epibiont *Epistylis* sp. exposed to different temperature–salinity combinations for 24 hours. Error bars represent standard error, n=10.

**Salinity-turbidity effect on *Epistylis* sp. survivorship**

Salinity interacted with turbidity to affect epibiont survivorship in both experiments (Experiment 1:  $F_{12,189} = 3.45$ ,  $p < 0.001$ ; Experiment 2:  $F_{12,189} = 2.24$ ,  $p < 0.05$ ). For Experiment 1, the Tukey post hoc test revealed that survivorship at the lowest salinity (zero) and highest turbidity (1000 NTU) and at the salinity of 5 and turbidity of 50 NTU accounted for most of the differences, as these combinations were each significantly different to 8 other combinations. Salinity-turbidity combinations accounting for the significant difference were more numerous in the second experiment, as epibiont survivorship in 6 salinity–temperature combinations was different to survivorship in 6–8 other combinations.

On average, survivorship at the highest turbidity level (1000 NTU) was lower than that at the other turbidity treatments across the salinity gradient (Figure 5.2). At this turbidity level, there were wide fluctuations in survivorship across the entire salinity gradient. These fluctuations also differed with each experiment. In Experiment 1, the lowest survivorship was at the salinity of 0 followed by 25. Survivorship was highest at 10 and 20. In Experiment 2, survivorship was lowest at the salinity of 5, followed by 20 and 25. Survivorship was highest at the salinities of 0 and 15 (Figure 5.2).

Although survivorship was higher at 50 NTU, there was also no clear trend in survivorship across the salinity gradient. However, across the salinity gradient and in each experiment, the fluctuations in this turbidity treatment were not as pronounced as those at 1000 NTU. In contrast, at 250 NTU survivorship was substantially lower at the two highest salinities (20 and 25) in both runs of this experiment. The trend seen across the salinity gradient at 250 NTU is similar to that found in the salinity–temperature experiment, as here too survivorship was generally lowest at the two highest salinities.

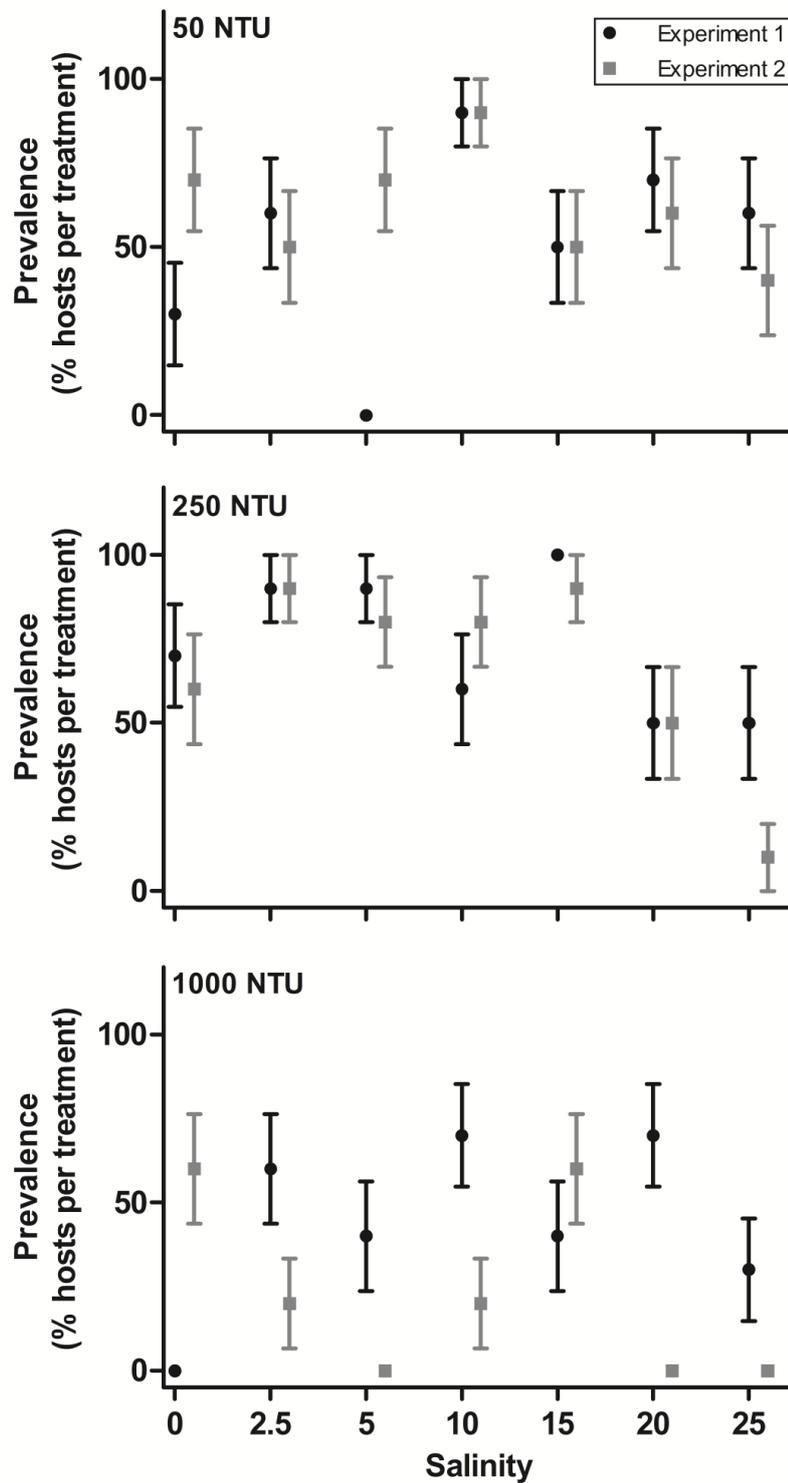


Figure 5.2: Survivorship of the epibiont *Epistylis* sp. exposed to different turbidity–salinity combinations for 24 hours. Error bars represent standard error, n=10.

## DISCUSSION

The aim of this study was to determine the effects of salinity, temperature and turbidity on the mortality rate of the ciliate *Epistylis* sp. While temperature had no effect on *Epistylis* sp., this epibiont was unable to tolerate salinities greater than 30 and there was high mortality in treatments between 20 and 25. In contrast, most of the epibionts exposed to the treatment of 15 and below (up to fresh conditions) were able to survive for 24 hours. Salinity interacted with turbidity to affect the survivorship of *Epistylis* sp., with the influence of turbidity becoming particularly apparent at the highest level (1000 NTU). As in the temperature-salinity experiments, survivorship was generally low at the highest salinity level (25).

Although turbidity is generally considered harmful to biota, there are several ways in which some species can exploit or withstand this condition. These include evasion of visual predators, gaining competitive advantage over less tolerant taxa and utilizing as a food supplement organic matter mixed with or attached to the suspended sediment (Vinyard and O'Brien 1976; Arruda et al. 1983; Henley et al. 2000). The study detailed in Chapter 4 was aimed at determining the effects of turbidity on the attachment success of the telotroch stage of *Epistylis* sp. to *P. stuhlmanni*. To test if the epibiont derived benefits from the organic matter in the silt, separate experiments were conducted, one using natural (i.e. nutritious) St Lucia silt and another using silt that had been combusted to burn off organic matter. Results of the inorganic silt experiments showed a negative relationship between turbidity and epibiont attachment success across the turbidity gradient 8–1200 NTU. At turbidities of 1000 NTU and above, virtually no epibionts were present in the inorganic experiments. The natural silt experiments however showed that *Epistylis* sp. was most successful in establishing itself in the turbidity range 250–500 NTU.

Those findings suggest that *Epistylis* sp. can exploit natural silt but only up to approximately 500 NTU. The findings in Chapter 4 and those of the current study imply that intermediate levels of nutritious silt are beneficial to *Epistylis* sp. in its free-swimming, host-seeking form and in its attached form, as in this study the average survivorship of attached *Epistylis* sp. was highest at 250 NTU. However, this high survivorship was roughly the same as that found in the temperature-salinity experiments. It may be that the nutrition derived from the algae provided in the temperature-salinity experiments was equivalent to that in silt of the 250 NTU

treatment, and that at 1000 NTU the benefits were not enough to buffer the negative effects of the actual silt.

Of the species tested, the copepod *P. stuhlmanni* appears to be the most turbidity-tolerant zooplankton species in St Lucia (Jones et al. 2015 and references therein). Jones et al. (2015) experimentally tested the tolerance of this species and found that between 80 and 100 % of individuals were able to survive past the 96-hour mark in the turbidity treatment of 500 NTU. The fact that both *Epistylis* sp. and *P. stuhlmanni* survive best in the intermediate turbidity range (250–500 NTU), even when collected at much lower turbidities in the field, is interesting and may have an evolutionary basis. *Pseudodiaptomus stuhlmanni* can exploit higher turbidities and survives over a longer duration than *Epistylis* sp. in sub-optimal turbidities. The epibiotic relationship between these two taxa is governed by the abundance of *P. stuhlmanni*, as in host density-dependent experiments Jones et al. (2016b [Chapter 2]) found a higher prevalence in treatments with more *P. stuhlmanni* individuals. The tolerance of the turbidity range 250–500 NTU by *Epistylis* sp., even under laboratory conditions where host numbers are controlled, may still reflect not so much a preference for this turbidity range but rather the results of co-evolution with the host. If under field conditions *P. stuhlmanni* attains high densities at intermediate turbidities, it would be advantageous for the density-dependent and host-specific *Epistylis* sp. to adapt to these particular turbidity levels, so as to exploit substrate availability.

The findings of this study provide insight into whether *Epistylis* sp. can interact with environmental variables to affect *P. stuhlmanni* in St Lucia. As discussed above, the turbid conditions that favour the presence of *Epistylis* sp. (250–500 NTU, natural silt) also favour *P. stuhlmanni* (Jones et al. 2015). *Epistylis* sp. can only interact with turbidity to affect *P. stuhlmanni* at or below this optimal turbidity range, as this epibiont is less tolerant of the higher turbidity levels than *P. stuhlmanni*. As high cover of *P. stuhlmanni* by *Epistylis* sp. is associated with low *P. stuhlmanni* survivorship (Jones et al. 2016b [Chapter 2]), between the turbidity range 250–500 NTU growth and reproduction of *Epistylis* sp. is expected to reduce the longevity of *P. stuhlmanni*. At the lower turbidities ( $\leq 50$  NTU), direct competition between epibiont and host for food might negatively affect the host, but this would likely not affect host survivorship if epibiont cover remains low (although negative impacts may manifest in other ways [Chapter 3]).

Whereas *P. stuhlmanni* is tolerant of salinity up to 65 (Grindley 1976; 1982), *Epistylis* sp. cannot withstand salinities higher than 25 and therefore cannot interact with these high salinities to affect *P. stuhlmanni*. Below the salinity of 20, *Epistylis* sp. could negatively affect *P. stuhlmanni* abundance only if other factors such as food availability are favourable enough to enable this epibiont to attain high cover on *P. stuhlmanni*.

In this study, no relationship was found between temperature and epibiont survivorship. Temperature may however affect epibiosis indirectly through its effect on the growth and reproduction of host zooplankton. Ciliate epibionts reproduce only while attached to their hosts (Allen et al. 1993; Gaiser and Bachmann 1993). Temperature dictates instar duration of zooplankton (Barea-Arco et al. 2001 and references therein) and higher temperatures generally cause shorter zooplankton intermoult duration. Therefore, the establishment and growth of ciliate epibionts on zooplankton hosts may be hindered by the frequent moulting caused by high temperatures, as while the host moults the epibionts are forced to detach and assume their host-seeking form. The St Lucia population of *Epistylis* sp. attaches predominantly to adult *P. stuhlmanni* (Chapter 2). As St Lucia is located in the subtropics, the typically high temperatures might explain the preference for the adult forms, as they provide a more stable substrate. In this way, temperature (or at least the range used in this study) may not affect *Epistylis* sp. *per se*, but rather affect the interaction between this epibiont and its host.

In conclusion, this study has revealed that the survivorship of the St Lucia population of the ciliate epibiont *Epistylis* sp. is unaffected by temperature but negatively affected by high salinity and high turbidity. As the host species *P. stuhlmanni* is more tolerant of the tested sub-optimal conditions, high salinity and turbidity are expected to modulate epibiosis through negative effects on the epibiont's survivorship. Therefore, any interaction between *Epistylis* sp. and these parameters is expected to be substantially beneficial to this epibiont only in conditions that are well tolerated and/or exploited by both epibiont and host. Although the information gained in this study is useful for predicting the short-term response of *Epistylis* sp. to salinity, temperature and turbidity, long-term monitoring is required to supplement these findings and capture the optimal conditions of this ciliate in St Lucia. This monitoring would also aid in gaining a better understanding of the interaction among *Epistylis* sp., *P. stuhlmanni* and prevailing environmental conditions.

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## Dynamics of the peritrich epibiont *Epistylis* sp. and its host, the calanoid copepod *Pseudodiaptomus stuhlmanni* in the St Lucia Estuary, South Africa

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### ABSTRACT

A study was undertaken in the St Lucia Estuary, aimed at determining the relationship between the occurrence of the epibiotic peritrich *Epistylis* sp., its calanoid host *Pseudodiaptomus stuhlmanni* and environmental conditions. Monthly surveys were conducted throughout 2016 at two stations within St Lucia (Dredger Harbour and Sugarloaf Jetty). Host abundance and epibiont prevalence and cover were recorded throughout the study period. Only mature stages of *P. stuhlmanni* were observed hosting epibionts and in general, epibiont prevalence and cover was higher on females than on males. Epibionts attached all along the dorsal surface, with the metasome being the dominant site of attachment, while no epibionts were observed on the ventral surface. The abundance of *P. stuhlmanni* was correlated with salinity at both sites as well as with particulate organic matter at Sugarloaf. No correlation was found between epibiont prevalence and host abundance, as well as between prevalence and other variables, which suggests that a more complex interaction of variables governs this epibiosis. However, as the abundance of *P. stuhlmanni* was uncharacteristically low throughout 2016, replication of this study under a different hydrological phase is recommended so that the implications of epibiosis can be fully assessed.

**Keywords:** ciliate epibiosis, environmental drivers, site-specificity, zooplankton

### INTRODUCTION

Microscopic organisms such as algae, bacteria, and ciliates are commonly found living on the surface of crustaceans in an ectosymbiotic relationship known as epibiosis (Green and Shiel 2000; Utz and Coats 2005; Souissi et al. 2013). Some studies have shown that epibionts have the potential to affect the population dynamics of their hosts by influencing such processes as feeding, locomotion, predator evasion and mating, often to the detriment of the host (Weissman et al. 1993; Gilbert and Schröder 2003; Visse 2007; Bielecka and Boehnke 2014).

In spite of the widespread nature of epibiosis, there is limited information in the primary literature on the occurrence of epibiosis in crustacean communities dwelling in African waters. Further, the existing studies that do report occurrence do not provide information on host-epibiont dynamics over time (e.g. Basson and Van As 1991; West et al. 2016). This information is important to understand the ecology of the epibiont, but also in cases where the epibiont is able to substantially affect its host.

*Epistylis* sp. is a sessilid peritrich ciliate frequently attached to *Pseudodiaptomus stuhlmanni*, a numerically dominant calanoid copepod from the St Lucia Estuary (Figure 1.1), Africa's largest estuarine lake and a Ramsar Wetland of International Importance (Begg 1978; Whitfield et al. 2013). Although St Lucia has three dominant copepod species, *Epistylis* sp. displays host specificity as this peritrich attaches only to *P. stuhlmanni* (Jones et al. 2016b; [Chapter 2]). Further, *P. stuhlmanni* individuals with a high epibiont cover (percentage of host body covered by epibionts) have a lower longevity than non-hosting counterparts (Chapter 2). *Epistylis* sp. also reduces the nutritional state of hosting *P. stuhlmanni* (Chapter 3). Therefore, *Epistylis* sp. has the potential to influence the population dynamics of *P. stuhlmanni* and to alter the zooplankton community structure in St Lucia.

Physico-chemical conditions and food availability are known to modulate epibiosis (Green and Shiel 2000). This study reports the epibiont-host population dynamics in relation to prevailing environmental conditions over a one year period (January– December 2016) in the St Lucia Estuary.

## **MATERIALS AND METHODS**

### **Sample collection**

Monthly zooplankton samples were collected from January until December 2016 at two stations within the St Lucia Estuary: Dredger Harbour and Sugarloaf Jetty (Figure 1.1). These two sites were chosen due to their close proximity and the different conditions prevailing in each. Dredger Harbour is ~5.5 km from the estuary mouth and is connected to the estuary through a narrow opening. This site experiences relatively stable water quality conditions. Sugarloaf Jetty is only ~70 metres from the estuary mouth and is more exposed to wind action and periodically affected by incoming fresh- and seawater.

Duplicate zooplankton samples were collected per station on each sampling occasion. To obtain each sample, 50 L of water was poured through a 100  $\mu\text{m}$  mesh. The samples were then fixed with 5 % Bouin's fluid (Utz and Coats 2005). In the laboratory, the samples were examined in their entirety under a Kyowa SDZ dissecting microscope (40  $\times$ ). All zooplankton taxa captured in the samples were examined for epibiont attachment; however, only *P. stuhlmanni* adults were observed hosting epibionts. Therefore, the focus of this study is only on mature stages of this species. To verify the identity of the epibionts, eight epibiont-hosting *P. stuhlmanni* individuals were randomly selected from each sample with epibionts and further examined at 100  $\times$  magnification. When samples that had less than eight infested individuals, all hosting individuals were examined. The epibionts were all verified to be *Epistylis* sp.

Prevalence (percentage of host species with epibionts) and cover were recorded. In order to assess epibiont cover, *P. stuhlmanni* individuals were ranked into three groups: copepods with a low cover (< 20 % body cover); copepods with a medium cover (20–40 % body cover) and epibiont-hosting copepods with a high epibiont cover (> 40 % body cover). Epibiont-hosting copepods were examined for sex and as some epibionts have been found to attach to specific body parts, the position of attachment was also recorded.

### **Environmental conditions**

Rainfall data were obtained from the Ezemvelo KwaZulu-Natal Wildlife conservation authority. Salinity, temperature and turbidity were measured with an ATAGO S/Mill-E hand refractometer, a mercury thermometer, and a HACH2100Qis turbidity instrument, respectively. At each site and on each sampling occasion, duplicate water samples were collected in order to measure the concentration of phytoplankton as well as total suspended solids (TSS) and particulate organic matter (POM). The size class distribution of phytoplankton was determined by sequentially filtering duplicate 150 mL sub-surface samples of water through 20, 2 and 0.7  $\mu\text{m}$  meshes. These filters were then placed in vials with 6 mL of 90 % acetone and placed in a freezer for 48 hours of cold, dark pigment extraction. Phytoplankton biomass ( $\text{mg pigm m}^{-3}$ ) was determined fluorometrically using a Turner Designs 10-AU non-acidification system (Nozais et al. 2001).

The concentrations of TSS ( $\text{mg L}^{-1}$ ) and POM ( $\text{mg L}^{-1}$ ) were determined by filtering 150 mL of estuarine water through pre-combusted (6 hours, 420°C in a muffle furnace) Whatman GF/F filters. Once oven-dried (48 hours, 60°C), the filters were weighed to 0.1 mg using a Shimadzu

AUW220D Uni Bloc balance, combusted (6 hours, 420°C) and re-weighed in order to calculate TSS and POM (Carrasco et al. 2007).

### **Statistical analyses**

Most of the data violated the assumptions of parametric tests, including normality and homoscedasticity. Therefore, all statistics analyses were conducted with non-parametric alternatives. Epibiont prevalence and *P. stuhlmanni* abundance in relation to each other and to environmental variables were tested with Spearman Rho correlations. The size of the *Epistylis* sp. in St Lucia is within the size range that typically ingests nano- and pico-particles (average peristomial lip width of 20 µm [Chapter 1]; also see Utz and Coats 2005 and references therein). Therefore, the phytoplankton biomass data used to analyze correlations with epibiont prevalence were those corresponding to algae retained in the 2 and 0.7µm filters. The data used for correlations with *P. stuhlmanni* abundance included algae retained in all three mesh sizes (20, 2 and 0.7 µm).

The Mann-Whitney U test was used to test for differences in prevalence between the two sites (Dredger Harbour and Sugarloaf Jetty) as well as between male and female adults. To test for differences in the frequency of occurrence of the three groups of epibiont cover (low, medium and high) at each site as well as between males and females, the Chi Square test was used. To carry out the analyses, copepods with low, medium and high cover were coded as 0, 1 and 2, respectively. Chi square analysis was also used to test for preference of the Dredger Harbour epibionts for specific body sites or combinations thereof. For analysis, attachment to the whole body was coded as 0: attachment to the prosome (cephalosome [including antennae] and metasome); as 1: to the cephalosome and urosome; as 2: to the metasome and urosome; as 3: to only one body segment; as 4: cephalosome; 5: metasome; and 6: urosome.

## **RESULTS**

### **Environmental conditions**

St Lucia was experiencing a wet phase throughout 2016, with rainfall generally above average (average of  $138 \pm 69$  mm throughout the year). The months with the highest and lowest rainfall were July (313 mm) and August (42 mm), respectively. Due to freshwater inflow, salinity was generally low throughout the year, averaging  $2.67 \pm 4$  at the Dredger Harbour and  $2.08 \pm 4.23$  at the Sugarloaf Jetty throughout the year. The month with the highest salinity was October (13

and 15 at Dredger Harbour and Sugarloaf Jetty, respectively). Apart from the months of October, January and February, both sites had very low salinity, between 0 and 2 (Figure 6.1).

Water temperature ranged from 19 to 33 °C and 20 to 33 °C at Dredger Harbour and Sugarloaf Jetty, respectively. Unlike the other parameters, there was a pattern in temperature values throughout the year, as the lowest values fell in the last 6 months of the year. The average temperature from January to June was  $28 \pm 3.11$  °C at Dredger Harbour and  $28.5 \pm 2.63$  °C at Sugarloaf Jetty. From July to December, the average temperature was  $24.2 \pm 3.02$  °C at Dredger Harbour and  $25 \pm 2.77$  °C at Sugarloaf Jetty (Figure 6.1).

Turbidity was highly variable, averaging  $278 \pm 286$  NTU and  $293 \pm 266$  NTU at Dredger Harbour and Sugarloaf Jetty, respectively. Turbidity was highest in August (Dredger Harbour:  $1047 \pm 53$  NTU; Sugarloaf Jetty:  $979 \pm 3.5$  NTU) and lowest in May (Dredger Harbour:  $50 \pm 2$  NTU; Sugarloaf Jetty:  $65.5 \pm 3.5$  NTU). The highest TSS concentration coincided with the highest turbidity in August (Dredger Harbour:  $1110 \pm 36.1$  mg.L<sup>-1</sup>; Sugarloaf Jetty:  $633 \pm 36.2$  mg.L<sup>-1</sup>). Particulate organic matter constituted only 15 % (Dredger Harbour) and 26 % (Sugarloaf Jetty) of the TSS during this month, meaning that the turbidity was mainly due to inorganic material (Figure 6.1).

The average TSS throughout the year was  $284 \pm 355$  mg.L<sup>-1</sup> at Dredger Harbour and  $198 \pm 146$  mg.L<sup>-1</sup> at Sugarloaf Jetty. The POM proportion was only  $58.2 \pm 59.3$  mg.L<sup>-1</sup> at Dredger Harbour and  $48.8 \pm 40$  mg.L<sup>-1</sup> at Sugarloaf Jetty. Throughout the year, POM contributed ~20 and 25 % to the total TSS at Dredger Harbour and Sugarloaf Jetty, respectively. At Dredger Harbour, phytoplankton concentration ranged between  $4.59 \pm 1.7$  mg.m<sup>-3</sup> in September and  $47 \pm 1.37$  mg.m<sup>-3</sup> in October. At Sugarloaf Jetty, phytoplankton concentration was also lowest in September ( $8.2 \pm 3.11$  mg.m<sup>-3</sup>) and highest in November ( $43 \pm 4.6$  mg.m<sup>-3</sup>; Figure 6.1).

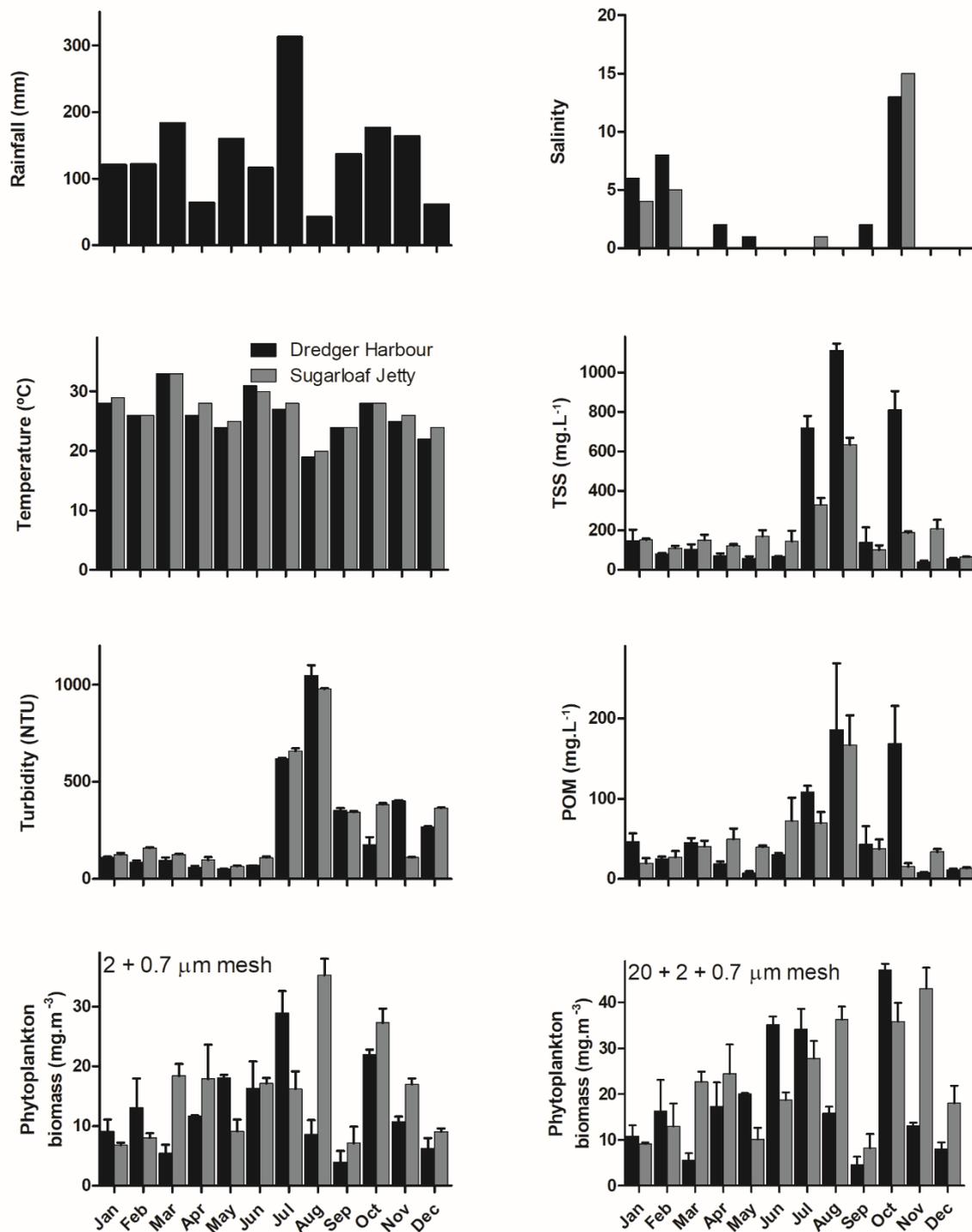


Figure 6.1: Environmental parameters measured throughout 2016. Error bars represent standard deviation.

### ***Pseudodiaptomus stuhlmanni* abundance and sex ratio**

Dredger Harbour had a higher overall abundance than Sugarloaf Jetty, accounting for ~ 72 % of the adult *P. stuhlmanni* population throughout the year. This difference was significant (Mann-Whitney U = 100,  $p < 0.0001$ ). At Dredger Harbour, the highest abundance of *P. stuhlmanni* was in January ( $183 \pm 1.7 \text{ ind.m}^{-3}$ ) and the lowest in June ( $21.6 \pm 1.45 \text{ ind.m}^{-3}$ ; Figure 6.2 A). The highest abundance at Sugarloaf Jetty was also in January ( $59.6 \pm 0.318 \text{ ind.m}^{-3}$ ) and the lowest in July ( $10.93 \pm 0.46 \text{ ind.m}^{-3}$ ; Figure 6.2 C).

At both sites, females generally outnumbered males. Overall, females accounted for ~ 55 and 58 % of the *P. stuhlmanni* population at Dredger Harbour and Sugarloaf Jetty, respectively. At Dredger Harbour, the abundance of males exceeded that of females in four months (March–May and September; Figure 6.2 A). At Sugarloaf Jetty, the abundance of males only exceeded that of females in February (Figure 6.2 C). There was however no statistical difference between the abundance of the sexes at either site (Dredger Harbour: U = 236,  $p > 0.05$ ; Sugarloaf Jetty: U = 202,  $p > 0.05$ ).

### ***Epistylis* sp. prevalence**

At both sites, the abundance of epibiont-hosting copepods was significantly lower than that of non-hosting individuals (Dredger Harbour: U = 48,  $p < 0.0001$ ; Sugarloaf Jetty: U = 0,  $p < 0.001$ ). Dredger Harbour had a higher overall epibiont prevalence, with nearly 16 % of the copepods hosting epibionts throughout the year, as opposed to 5.24 % at Sugarloaf Jetty. However, the Mann-Whitney U test revealed no significant difference in epibiont prevalence between the sites (U= 254;  $p > 0.05$ ).

The prevalence of *Epistylis* sp. at Dredger Harbour was not related to the abundance of *P. stuhlmanni*. Whereas the abundance of the copepods was highest in January, prevalence was highest in April ( $66.2 \pm 2.22 \%$ ) and *Epistylis* sp. was virtually absent across the May–December period (Figure 6.2 B). A similar trend was observed at Sugarloaf Jetty, where low prevalence was recorded between May and December 2016 (Figure 6.2 D).

Epibiont prevalence on females throughout the year was higher than on males at both sites (Dredger Harbour females: 20 %, males: 6 %; Sugarloaf Jetty females: 8.5 %, males: 4.26 %),

but no significant difference was found between the sexes (Dredger Harbour:  $U = 246$ ;  $p > 0.05$ ; Sugarloaf Jetty:  $U = 224$ ,  $p > 0.05$ , Figure 6.2 B and D).

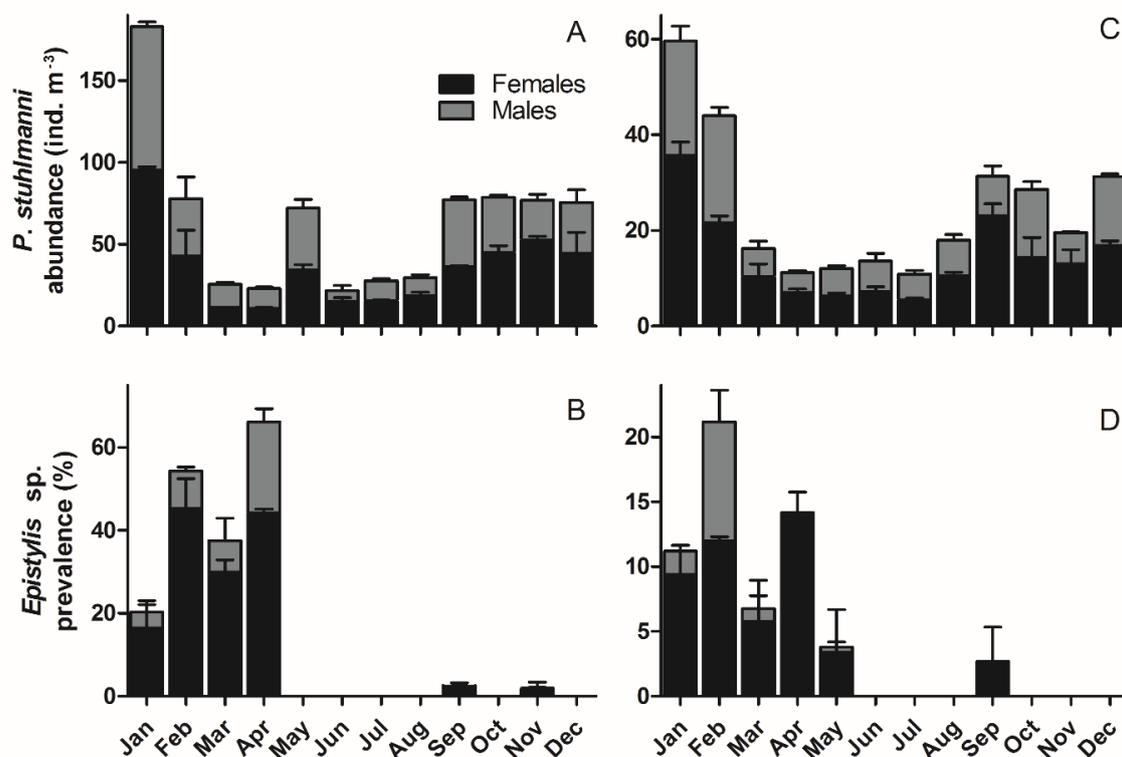


Figure 6.2: Abundance of the host copepod *Pseudodiaptomus stuhlmanni* in relation to prevalence of the epibiont *Epistylis* sp. from January to December 2016. 6.2 A and C show contribution of females and males to the adult population at Dredger Harbour and Sugarloaf Jetty, respectively. 6.2 B and D show contribution of hosting females and males to the overall prevalence at Dredger Harbour and Sugarloaf Jetty, respectively. Error bars represent standard deviation. Note the different scales on the y-axes.

### *Epistylis* sp. cover

Chi-Square analysis revealed a significant difference in the frequency of occurrence of the three levels of epibiont cover on *P. stuhlmanni* within both site (Dredger Harbour:  $\chi^2 = 1496$ ,  $p < 0.001$ ; Sugarloaf Jetty:  $\chi^2 = 142$ ,  $p < 0.001$ ). At Dredger Harbour, copepods that accounted for the largest proportion of the hosting *P. stuhlmanni* population were those with a low cover (56.2 %), followed by copepods with medium cover (36.2 %). Copepods with a high epibiont cover were the least numerically dominant (7.62 %). At Sugarloaf Jetty, copepods with a medium cover contributed the most to the overall *P. stuhlmanni* host population (45.9 %).

Copepods with a low and high epibiont cover contributed 40.4 and 13.7 % respectively to the *P. stuhlmanni* host population.

Chi-Square analysis also revealed a significant difference in the frequency of occurrence of the three groups of epibiont cover on male and female *P. stuhlmanni* at both sites (Dredger Harbour:  $\chi^2 = 50$ ,  $p < 0.001$ ; Sugarloaf Jetty:  $\chi^2 = 8.44$ ,  $p < 0.05$ ). While females dominated across all three groups at both sites, this disparity was especially pronounced for high epibiont cover, as it was mainly observed on females (Table 6.1).

Table 6.1: Proportion of hosting male and female *Pseudodiaptomus stuhlmanni* with low, medium and high epibiont cover from January to December 2016.

Epibiont cover	Dredger Harbour			Sugarloaf Jetty		
	Male	Female	Total	Male	Female	Total
Low	12.5	43.7	56.2	9.67	30.7	40.4
Medium	7.11	29.1	36.2	14.4	31.5	45.9
High	0.406	7.21	7.62	2.64	11.1	13.7

### *Epistylis* sp. body site-specificity

*Epistylis* sp. was never observed on the legs or ventral region. This epibiont attached to all sites along the dorsal surface and sometimes to the sides of the body; however, analysis revealed a significant difference in body site-specificity ( $\chi^2 = 2382$ ,  $p < 0.001$ ). The dominant attachment site was the metasome as 86 % of all infested individuals hosted epibionts either on the metasome alone or on the metasome in combination with other sites. The cephalosome was the second most dominant attachment site. Although a significant difference was found in attachment site specificity between males and females ( $\chi^2 = 77.7$ ,  $p < 0.001$ ), for both sexes the preferred site was the metasome and the least utilized was the urosome (Table 6.2).

Table 6.2: Epibiont occurrence (%) on the body of the host *Pseudodiaptomus stuhlmanni* at Dredger Harbour.

	Prosome	Cephalosome		Metasome	Cephalosome	Metasome	Urosome
	+ urosome	Prosome	+ urosome	+ urosome			
Male	0.668	7.5	0.048	5.82	2.41	3.15	0.382
Female	9.39	23.1	0.382	20.8	9.36	15.6	1.36
Total	10.1	30.6	0.43	26.6	11.8	18.8	1.74

### Correlation analysis

Spearman Rank Correlation analysis revealed a positive correlation between the abundance of *P. stuhlmanni* and salinity at Dredger Harbour and Sugarloaf Jetty. The abundance of this copepod correlated with particulate organic matter concentration at Sugarloaf Jetty (Table 6.3). There was no correlation between the abundance of *P. stuhlmanni* and epibiont prevalence at either site. There was also no correlation between prevalence and any of the other variables (rainfall, temperature, salinity, turbidity, TSS, POM and phytoplankton density) at either site (Table 6.3).

Table 6.3: Spearman Rho Correlation analyses between the prevalence of *Epistylis* sp. and the abundance of *Pseudodiaptomus stuhlmanni*. Copepod abundance and epibiont prevalence were also analysed for correlations with other variables (n = 24 in all cases).

		Epibiont prevalence	Host abundance	Rainfall	Salinity	Temperature	Turbidity	TSS	POM	Phytoplankton
<b>Dredger Harbour</b>										
Epibiont prevalence	Correlation coefficient	—	-0.016	-0.036	0.402	0.271	-0.372	-0.08	-0.118	-0.357
	p-value	—	0.94	0.867	0.051	0.2	0.073	0.711	0.584	0.086
Host abundance	Correlation coefficient	-0.016	—	0.073	0.476	-0.231	0.203	-0.045	-0.099	-0.194
	p-value	0.94	—	0.734	<b>0.019</b>	0.277	0.34	0.835	0.645	0.364
<b>Sugarloaf Jetty</b>										
Epibiont prevalence	Correlation coefficient	—	0.195	-0.087	0.219	0.27	-0.332	-0.35	-0.228	-0.387
	p-value	—	0.362	0.687	0.304	0.202	0.113	0.094	0.285	0.062
Host abundance	Correlation coefficient	0.195	—	-0.201	0.432	-0.237	0.281	-0.308	-0.601	-0.27
	p-value	0.362	—	0.347	<b>0.035</b>	0.265	0.183	0.143	<b>0.002</b>	0.203

## DISCUSSION

Epibiont prevalence is typically high when host species are abundant (Chiavelli et al. 1993; Mohlemberg and Kaas 1990; Xu 1992), as transmission is dependent on encounter rates with the host. In contrast to observations in previous years (Carrasco et al. 2010; Carrasco and Perissinotto 2015), the abundance of *P. stuhlmanni* was generally low throughout 2016. Unfortunately, the zooplankton sampled prior to 2016 were collected at different sites within St Lucia, at wider-spaced intervals (~ 3-month intervals) and using a different sampling technique. Therefore, these cannot be quantitatively compared with the samples from the current study.

The purpose of the free-swimming telotroch stage of peritrichs is to find attachment substrates. Thus telotrochs are short-lived as they either attach or die if unable to find hosts timeously (Utz and Coats 2008). The low prevalence even in months when *P. stuhlmanni* was relatively abundant might mean that there is a threshold host availability and thus encounter rate below which *Epistylis* sp. is unable to successfully transfer from one host to another. This would explain the lack of a correlation between epibiont prevalence and host abundance. Even

copepods with a low epibiont cover generally hosted more than one attached colony, which suggests that when zooids divided, transmission occurred from one site to the next on the same host individual.

That the population of this copepod was not correlated with the prevalence of *Epistylis* sp., coupled with the low occurrence of high epibiont cover throughout 2016, suggests that epibiosis is not among the factors driving the *P. stuhlmanni* population. This might be the case only under the conditions prevailing in 2016 in the southern parts of St Lucia (low salinity, predation pressure, dominance of freshwater taxa). Replicating the present study during a different hydrological phase would help determine if the weak relationship between *P. stuhlmanni* and *Epistylis* sp. holds true under different conditions, or if the implication of epibiosis for these organisms is dependent on environmental context.

The study reported in Chapter 5 revealed that *Epistylis* sp. is tolerant of limnetic to mesohaline conditions, with no particular salinity preference between 0 and 15. This epibiont was less tolerant of higher salinities ( $\geq 20$ ), with 0 % survivorship at 30 and above. The salinity at both sites throughout 2016 was between 0 and 15, which might be the reason why salinity was not picked up as a driving factor. Likewise, temperature was not correlated with epibiont prevalence, which also corroborates the findings of Chapter 5.

Jones et al. (2016a) compared the zooplankton community structure of the Narrows region of St Lucia (Figure 1.1) before and following a 2014 flood event that left the southern lakes of St Lucia highly turbid and limnetic to oligohaline. A substantial decline in the abundance of *P. stuhlmanni*, a disappearance of the other resident estuarine zooplankton, and a dominance of freshwater cyclopoids and cladocerans after the flood event were recorded. Jones et al. (2016a) suggested sub-optimal conditions, including competitive interactions with the freshwater taxa, as reasons for the low *P. stuhlmanni* abundance. Although the data is not presented in this study, the community structure throughout 2016 and as of November 2017 at Dredger Harbour and Sugarloaf Jetty resembled that found post the 2014 flood event, as it was dominated by these freshwater taxa. Another biotic factor not examined in this study which could have affected the occurrence of *P. stuhlmanni* and *Epistylis* sp. in 2016 is predation pressure.

In January and February 2016, the zooplankton communities of Dredger Harbour and Sugarloaf Jetty were dominated by unidentified hydrozoan polyps. Micrographs of these hydrozoans revealed *P. stuhlmanni* in their guts (Supplementary material 6.1). Further, during the months

that the *P. stuhlmanni* population was recovering (August–December 2016), there was a population explosion of a flatworm belonging to the genus *Microstomum*. *Microstomum* sp. preyed heavily on *P. stuhlmanni* as most field-collected individuals had *P. stuhlmanni* in their guts (Supplementary material 6.2). It is possible that predation pressure also controlled the *P. stuhlmanni* population and prevented the re-establishment of *Epistylis* sp.

No copepodites hosted epibionts in this study. Although field-collected *P. stuhlmanni* copepodites are sometimes observed hosting *Epistylis* sp., adult hosts are always dominant numerically, irrespective of copepodite abundance. *Epistylis* sp. has also been shown to attach preferentially to adult *P. stuhlmanni* under laboratory conditions (Jones et al. 2016b [Chapter 2]). The frequent moulting of juvenile copepods make them an unstable and temporary substrate as it necessitates metamorphosis of epibionts into their telotroch form (Wahl 1989; Bickel et al. 2012). This process is also energetically costly and risky as telotrochs that fail to quickly find a host die (Utz and Coats 2008). Adult copepods not only provide a larger surface area for attachment but they do not moult, which makes them more reliable hosts. The preference for adults found in this study and which has also been found in other studies (e.g. Xu 1992; Willey and Threlkeld 1993; Cabral et al. 2017) was therefore not unexpected.

Although no significant difference was found in epibiont prevalence between male and female copepods at either site, prevalence was higher on females at both sites. There was a significant difference in epibiont cover between the two sexes, with females attaining a generally higher cover. Virtually no attachment was observed on the egg sacs and attachment sites on gravid females were the same as those on non-gravid individuals. As females outnumbered males at both sites, higher encounter rates between *Epistylis* sp. and females might be responsible for the higher prevalence and cover on this sex.

In a study of ciliate-zooplankton epibiosis in Chesapeake Bay, Utz and Coats (2005) examined site specificity of the epibionts *Epistylis* sp. and *Zoothamnium intermedium* on the host calanoids *Acartia tonsa* and *Eurytemora affinis*. After finding low epibiont densities on antennae and legs, these authors suggested that the mobility of these body parts made them unsuitable for epibiont attachment. In this study, while no epibionts were observed on legs, they were frequently recorded on antennae. Several studies have found a preference for attachment sites along areas that increase chances of food acquisition (Clamp 1973; Evans et

al. 1979; Bartlett and Willey 1998). It might be advantageous for *Epistylis* sp. to attach to antennae if this facilitates the interception of food particles while *P. stuhlmanni* is filter feeding.

*Epistylis* sp. also attached to the urosome but exhibited a preference for the dorsal surface of the metasome, with virtually no epibionts on the ventral side. The metasome has a larger surface area than the cephalosome and urosome and may therefore have higher encounter rates with telotrochs. The location of the metasome between the other two segments also makes it a more accessible attachment substrate for reproducing epibionts located on both the cephalosome and urosome.

There was a higher abundance of *P. stuhlmanni* and higher epibiont prevalence at Dredger Harbour compared to Sugarloaf Jetty. Sugarloaf Jetty is not only more exposed to wind action but is closer to the estuary mouth and therefore more prone to fresh- and saltwater intrusion. Although Dredger Harbour is also relatively close to the mouth and Sugarloaf Jetty, this site is more sheltered, with the residence time of water higher than that in Sugarloaf Jetty. The organisms at Dredger Harbour are therefore afforded better stability for establishment. Spatial heterogeneity is important in preventing local extinctions and the findings of this study suggest that in St Lucia, this heterogeneity biotically manifests in different community structures comprising the same taxa even at sites in close proximity to each other, such as Dredger Harbour and Sugarloaf Jetty. That 72 % of the *P. stuhlmanni* population was found at Dredger Harbour in 2016 implies that this site serves as a refugium in the mouth region during flooding, when plankton in the more exposed sites get flushed out of St Lucia.

Pauleto et al. (2009) studied epibiont communities in lentic and lotic environments and found higher prevalence in the former. Similarly, Cabral et al. (2017) found higher peritrich epibiont prevalence in Baia River, which is classified as semi-lotic with a low flow rate, compared to the lotic Parana and Ivinhema rivers, both of which are characterised by a high flow rate and low nutrient concentration. The higher prevalence of *Epistylis* sp. at Dredger Harbour despite lack of a correlation with host abundance suggests that this epibiont also directly benefits from the shelter provided by this site.

The interpretation of the findings of this study needs to account for the uncharacteristically low abundance of *P. stuhlmanni* at the studied sites throughout 2016, as this is not representative of the densities this species typically attains throughout St Lucia (Carrasco et al. 2010; Carrasco and Perissinotto 2015; Jones et al. 2016a). Higher host abundance may have exerted a

statistically detectable impact on epibiont prevalence as well as enabled higher epibiont cover to establish on *P. stuhlmanni*, both of which would have had different ecological implications. A future study in St Lucia should focus on analysing epibiosis under contrasting conditions and take into account a larger number of possible driving factors than was included in this study, such as oxygen, pH and if possible, predation and competitive interactions.

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## General Discussion

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Although epibiosis involving ciliates is widespread in aquatic environments and has been extensively documented, there is a geographical bias as the majority of studies have been based in the Western Hemisphere (see the reference list). As pertains to zooplankton hosts, calanoid copepods are the most widely reported, due to their overwhelming numerical dominance in the plankton (Turner 2004; Guo et al. 2012). The studies in the Western Hemisphere systems have predominantly revolved around host species belong to just two genera, *Acartia* sp. (mainly *A. tonsa*) and *Eurytemora* sp. (mainly *E. affinis*). For instance, 15 of the articles referenced in this thesis dealt with epibiosis involving either *Acartia* sp. or *Eurytemora* sp. The current study not only adds information to the under-studied African water bodies, but it also deals with epibiosis involving *Pseudodiaptomus* spp., a previously undocumented host genus.

Chapter 1 in this study provided the first record of the sessilid peritrich *Epistylis* sp. living as an epibiont of zooplankton in KwaZulu-Natal, South Africa. *Epistylis* is a cosmopolitan genus and has been documented living as an epibiont in North America (e.g. Utz and Coats 2005), South America (Cabral et al. 2017), India (Rajabunizal and Ramanibai 2011) and China (Wang et al. 2017). Utz and Coats (2005) recorded attachment of *Epistylis* sp. to the calanoid copepod *A. tonsa* in the Chesapeake Bay Estuary in America. Cabral et al. (2017) found *Epistylis* sp. attached to calanoid and cyclopoid copepods in the Parana River in Brazil. In Lake Velachery, India, Rajabunizal and Ramanibai (2011) recorded *Epistylis niagarae* attached to the cyclopoid copepod *Mesocyclops* sp.; and Wang et al. (2017) identified *Epistylis wuhanensis* in Hubei, China.

In Africa, Viljoen and Van As (1985) and Van As and Basson (1984) reported *Epistylis* sp. epibiotic on freshwater taxa (a crab and several fish species) in southern Africa. A peritrich epibiotic on crustaceans in Lake Albert, Uganda was identified as *Epistylis articulata* (Green 1965). However, Green (1965) based his characterisation on live observations only and judging by the morphology presented in his illustrations, the peritrich does not appear to belong to the genus *Epistylis*. The peristomial area in particular, which he described as “poorly developed”, does not have the well-defined everted peristomial lip characteristic of Epistylids.

Identification of peritrichs, including the majority of species belonging to the genus *Epistylis* is mainly based on morphological characteristics (Utz 2007 and references therein), but genetic

studies by Utz et al. (2010), Sun et al. (2016) and Wang et al. (2017) have revealed that the genus *Epistylis* is not monophyletic, as species morphologically belonging to this genus do not all cluster together. Utz et al. (2010) found that *Epistylis* sp. occurred in two different clades within the subclass Peritrichia. They also showed that different populations of the type specimen *E. plicatilis* from Brazil and China were not the same species according to their DNA sequences. Utz et al. (2010) suggested that the characters used to define the genus *Epistylis* are plesiomorphic within the subclass Peritrichia and that *Epistylis* consists of basal species all with retained ancestral features.

Similarly, Sun et al. (2016) found that members of *Epistylis* sp. morphologically resembling *E. galea* did not cluster together. Wang et al. (2017) recommended a taxonomic revision of this genus and suggested that *Epistylis* is composed of morphospecies, proposing convergent evolution as the mechanism behind the superficial similarity. This is in contrast to the basal species hypothesis of Utz et al. (2010). In light of this, DNA sequencing is essential for the species-level identification of the St Lucia population.

The SEM micrographs in Chapter 1 (Figure 1.6 A and B) revealed damage caused to the exoskeleton of *P. stuhlmanni* by the *Epistylis* sp. stalks, and also that *Epistylis* sp. acts as a vector of bacterial infection. This, coupled with the negative effects of *Epistylis* sp. found in Chapters 2 and 3 (i.e. compromised fitness of *P. stuhlmanni*) corroborate what is known about the effects of *Epistylis* spp. on their hosts (e.g. Turner et al. 1979; de Pádua et al. 2013; Gilbert and Schröder 2003; Visse 2007). The survivorship experiments (Chapter 2) revealed high mortality rates of hosts with high epibiont cover. The SEM images (Chapter 1, Figure 1.6 A and B), RNA:DNA ratio and RNA content results (Chapter 3) all revealed negative impacts even at low–medium cover.

The compromised physiology of epibiont-hosting *P. stuhlmanni* may manifest in reduced survivorship in the long term and/or reduced reproductive output if energy meant for growth and reproduction is diverted towards supporting the epibiont burden or healing the damaged exoskeleton. This study is the first to use the RNA:DNA ratio and RNA content to investigate the impacts of epibiont infestation. This is also the first study to link impacts of epibiosis at the individual level (survivorship experiments) to sub-organismal impacts (RNA:DNA ratio and RNA content).

In Chapter 2 the host-specificity of *Epistylis* sp. was tested, by exposing this epibiont to the dominant zooplankton species found in St Lucia. These were the calanoid copepods *P. stuhlmanni* and *Acatella natalensis*, the cyclopoid copepod *Oithona brevicornis* and the mysid *Mesopodopsis africana*. In monoculture and mixed culture experiments, *Epistylis* sp. only attached to *P. stuhlmanni* and predominantly to adult forms. This is consistent with previous observations (Jones et al. 2016a) and with the findings from field-collected monthly samples (detailed in Chapter 6). Compared with the other copepods exposed to *Epistylis* sp. in Chapter 2 (*A. natalensis* and *O. brevicornis*), *P. stuhlmanni* is long-lived and larger in size. Both factors might result in more encounters with the free-swimming stage of *Epistylis* sp. and, indeed, host density dependence experiments conducted in the same chapter showed a higher prevalence in treatments with high *P. stuhlmanni* numbers. However, encounter rates alone do not satisfactorily explain the specificity, as *Epistylis* sp. failed to attach to *O. brevicornis* and *A. natalensis* even in the absence of *P. stuhlmanni*. Other mechanisms, such as chemical and mechanical cues might be involved in locating and attaching specifically to *P. stuhlmanni*.

St Lucia is a dynamic system and prone to large fluctuations in turbidity and salinity (Whitfield and Taylor 2009; Carrasco et al. 2010; Zikhali et al. 2015). The tolerance of *P. stuhlmanni* tolerance for a wide turbidity (Jones et al. 2015) and salinity (Grindley 1976, 1982) range enable this species to persist year-round in St Lucia even under sub-optimal conditions (such as low salinity and/or high turbidity) which lead to the temporary disappearance of the other estuarine taxa (Jones et al. 2016a). This attribute, coupled with the high abundance and large size of *P. stuhlmanni*, makes this copepod the most practical and convenient attachment substrate and it is possible that *Epistylis* sp. has evolved to take advantage of this.

An interesting finding with the mixed culture experiments involving the mysid *Mesopodopsis africana* and *P. stuhlmanni* was the lack of attachment to not only *M. africana* but also to *P. stuhlmanni* during the incubation period. Epibionts are susceptible to predation by filter feeding zooplankton (Green 1974; Willey et al. 1990; Threlkeld and Willey 1993; Al-Dhaheri and Willey 1996). High clearance rates by *M. africana* may have reduced telotroch densities in these experiments. It is also possible that chemical cues released by *M. africana* deterred *Epistylis* sp. from both zooplankton species. This should be investigated in future research.

Biochemical assays should be incorporated more in ecological studies, as these indices can reveal the potential secondary production and nutritional state of organisms. Chapter 3

explored the sub-organismal impacts of epibiont attachment on *P. stuhlmanni*. A low RNA content and RNA:DNA ratio are stress indicators. In this study, epibiont-hosting and non-hosting organisms were divided into two groups and the nucleotide content of each determined. Both the RNA content and RNA:DNA ratio of the hosting group were lower than that of the non-hosting group. However, the difference was significant only for RNA content. This study is important as it highlights the energetic cost of epibiosis on host organisms. However, repeating this study either with a larger sample size or using a complementary extraction methodology would provide more conclusive evidence on the sub-organismal impacts of *Epistylis* sp..

Chapters 4 and 5 centred on experimental investigations of the physico-chemical drivers of *Epistylis* sp. presence and Chapter 6 focused on a field-based ecological approach. In the experimental studies, the attachment success of *Epistylis* sp. to *P. stuhlmanni* was negatively related to inorganic turbidity; and was highest in the turbidity range ~ 250–500 NTU in the natural silt (therefore organically rich) treatments (Chapter 4). The survivorship of *Epistylis* sp. was unaffected by temperature, positively affected by intermediate turbidity (natural silt) and negatively affected by high turbidity ( $\geq 1000$  NTU, natural silt); This epibiont had a low tolerance for salinities  $\geq 20$ , but showed no preference in the range 0–15. The field-based study, on the other hand, revealed no correlation between the prevalence of *Epistylis* sp. and these physico-chemical parameters.

There was also no correlation between prevalence and biotic factors (organic matter content and host abundance). During the field study, the zooplankton community in the sampled stations (Dredger Harbour and Sugarloaf Jetty) was dominated by freshwater taxa and although *P. stuhlmanni* persisted, the abundance of this copepod was low throughout 2016. In addition to the generally low salinity and high inorganic turbidity throughout 2016, biotic pressures, including competitive interactions with the freshwater taxa and predation pressure may have been responsible for the low abundances found throughout 2016. Samples of *P. stuhlmanni* sometimes exhibit very high *Epistylis* sp. prevalence, something that was observed at Charter's Creek and Dredger Harbour as recently as October 2017 (pers. observ.). I therefore recommend replicating this study, under a different hydrological phase and/or at the northern sites (e.g. Lister's Point, Figure 1.1), which have physico-chemical properties distinct from those of the southern sites sampled in this study.

## CONCLUSION

This study provides insights into a novel epibiotic relationship and does so from the perspective of both the epibiont and host, at different levels of biological organisation. Epibiosis involving zooplankton and peritrichs is widespread in estuaries, rivers and in the marine environment globally. Therefore, the findings presented in this thesis have global relevance and should help improve understanding of this relationship. As far as fitness tests go, researchers should be aware that the experimental approach, while important, may not be sufficient in detecting impacts on hosts. Biochemical indices provide essential information and organisms taken directly from the field can be used immediately, which eliminates confounding effects.

Given that even the mechanisms through which peritrichs, specifically *Epistylis* spp. attach to their hosts cause physical damage to the host body, peritrich epibionts should not be regarded as harmless commensals. Microscopical techniques such as SEM and possibly transmission electron microscopy (TEM) can also be used in the future, as micrographs obtained from these can directly show impacts on host individuals. The SEM approach can also show if individuals with no visible epibionts under normal light microscopy, had been previously hosting (see Chapter 1, Figure 1.6 D), something that has not been considered before in fitness tests. The prevalence and cover of *Epistylis* sp. was also low. Although there was no correlation between this epibiont and host abundance, epibionts need attachment substrates to establish, and the low *P. stuhlmanni* numbers may have made it difficult for *Epistylis* sp. to successfully transmit and proliferate.

Lastly, experimentally determining the environmental drivers of epibionts is essential as it will quickly give an indication of the tolerance range of these epibionts, information that may not be easily obtained from field-based observations. The epibiotic relationship is very complex and affected by many factors in the field, both biotic and abiotic. Replication of experiments (which was done in this study) and field observations (which should be done in future studies in St Lucia) will provide a more complete understanding of ciliate-zooplankton epibiosis.

## SUMMARY OF RECOMMENDATIONS FOR FUTURE RESEARCH:

- A) Species-level, genetics-based identification of the St Lucia population of *Epistylis* sp. This is currently underway and peritrich-specific primers are being used.

- B) Determine the reproduction rate of *Epistylis* sp. by isolating *P. stuhlmanni* hosts, recording the number of attached zooids, then counting the zooids at different time intervals.
- C) Determine the reproduction rate of *Epistylis* sp. by isolating *P. stuhlmanni* hosts, recording the number of attached zooids, then counting the zooids at various time intervals.
- D) Determine the predation pressure exerted by the St Lucia zooplankton (such as the mysid *M. africana*) on *Epistylis* sp. This could be done by recording feeding activities by the zooplankton through the use of a microscope fitted with a camera and visualizing software (see Chapter 1). A dye, such as the DAPI fluorescent stain could be used to stain the epibionts and thereafter track the feeding activity of the zooplankton.
- E) Perform more RNA extractions with copepods from St Lucia and nearby estuaries, in order to compare with the low RNA content and RND:DNA ratio found for *P. stuhlmanni* in this study.
- F) Monitor the dynamics of *Epistylis* sp. and *P. stuhlmanni* in St Lucia during a different hydrological phase and/or in the northern stations which have a physico-chemical environment distinct from the southern stations sampled in this study. As ciliates have a high turnover rate, the sampling could also be more intensive (eg. weekly) and/or seasonal (eg. daily over a two-week period per season).

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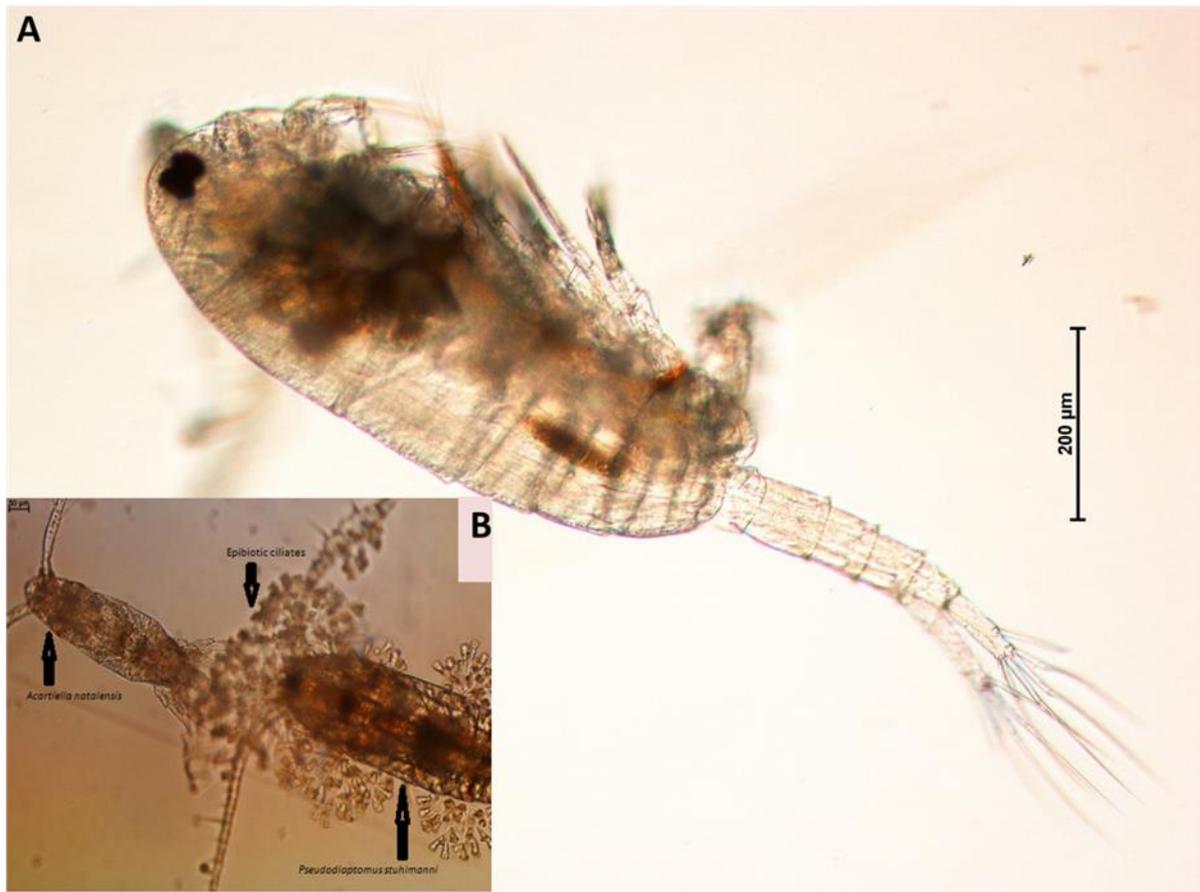
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## Supplementary Material

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### General Introduction



Supplementary material A: (A) The host species *Pseudodiaptomus stuhlmanni* and (B) the epibionts attached to *P. stuhlmanni* but not to *Acartiella natalensis*, another resident calanoid copepod.



Supplementary material B: the stations sampled during the course of this research within the St Lucia Estuary. A- Charter's Creek, B- the Narrows, C- Dredger Harbour, D- the Mouth, E- Sugarloaf Jetty.

## Chapter 1

Supplementary material 1.1:

### **Protargol Procedures for Routine Staining of Plankton Samples and Cultures (Montagnes and Lynn [1987], modified by Utz et al. [2008])**

#### **A. Day 1 (Do this in the afternoon, with last step completed between 4:00 and 4:30 PM)**

1. pre-wet 0.46 um Milipore filters (type HA, 25 mm diameter) in distilled water
2. melt test tube of 2.5% agar kept in refrigerator in almost boiling hot water

3. place several large glass coverslips and 22 mm square coverslips on a slide warmer (~ 55°C)
4. prepare 2% protargol solution by carefully sprinkling 0.2g protargol\*\* on surface of Columbia jar containing 10 mL tap water. **Do not mix, allow protargol to dissolve.** (Note: we get much better staining with tap water than with distilled water; however, the source of tap water can be critical & you may have to try several sources of water to get a good staining)
5. place pre-wet filter on wet filtering frit & turn on vacuum (< 200 mm Hg); attach filter tower with clamp, and turn off pump. This allows even contact of filter & frit and ensures even deposition of material across the filter; backing filter can be used to ensure even dispersal of sample if necessary
6. pour Bouin's fixed\* sample into 50 mL filter tower. If staining large volumes, settle sample in graduate cylinder for several days, siphon ~30 mL, mix and pour into tower followed by addition of three to four washes (5 mL each) of the graduate cylinder
7. turn on vacuum pump, allow all liquid to pass through filter, turn off pump, remove tower, and lift off filter
8. place filter, specimen side up, on large coverslip on the slide warmer and allow to dry on slide warmer for ~30 secs
9. place 1 drop of agar in center of 22 mm square pre-warmed coverslip (avoid air bubbles in agar drop)
10. pick coverslip with forceps and quickly invert so drop is suspended from coverslip
11. place coverslip on filter and allow agar spread out over filter surface.  
Coverslip can be carefully weighted down with forceps to avoid a thick layer of agar, but don't press hard!
12. remove large coverslip from warmer and allow to cool for ~1 min
13. cut away three edges of filter with a sharp single edged razor blade
14. grasp uncut edge of filter with forceps, carefully peel filter cover from 22 mm square coverslip and place in a Columbia jar of distilled water
15. clean several pieces of copper (one piece/filter, 5/Columbia jar of 0.005 thickness cut to 22 mm sq) by heating to a red hot in a flame & quickly

- immersing in a small beaker of 70% ethanol (procedure must be done in fume hood away from all flammable materials as the ethanol sometimes ignites)
16. place filters from step 14 in 0.5% potassium permanganate for 5 min
  17. rinse well with several distilled water changes until water no longer turns pink (takes ~5 min)
  18. place filters in 5% oxalic acid for 5 min
  19. rinse well with several distilled water changes over ~5 min
  20. protargol should be dissolved by now, if not wait until completely dissolved then mix well and place filters in staining jar (4 filters/jar not back to back)
  21. air dry pieces of clean copper & place one in front of each filter & one across the top of the jar, screw on caps & leave overnight at room temperature (Note: we get best staining after 15-16 hours in protargol solution)

**B. Day 2 (Start 8:00 – 8:30 AM)**

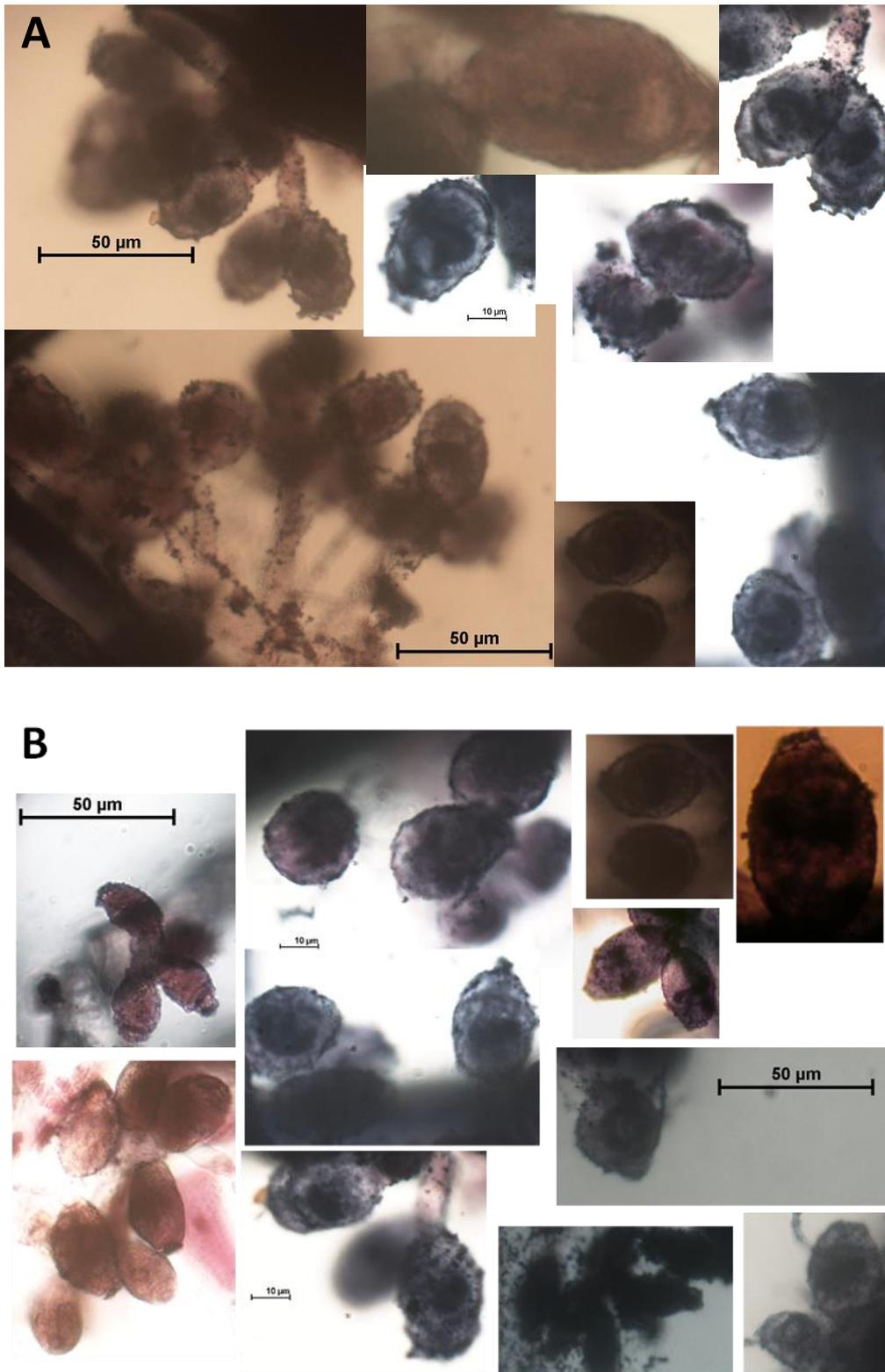
22. dissolve 0.1g hydroquinone in a 10 mL Columbia jar containing a solution of 4% sodium sulfite with 5% sodium carbonate (final: 1% hydroquinone)
23. remove filters from protargol and place in 4% sodium sulfite-5% sodium carbonate solution for ~5-10 secs
24. immediately transfer filters to hydroquinone for 5 min with frequent gentle agitation
25. rinse in distilled water for 3 min (**ONLY ONE EXCHANGE**); for dinoflagellate cultures rinse for only 1 min
26. transfer to a jar of 0.2% gold chloride for 15-20 secs. Filters will turn dark quickly and must be jiggled up and down to remove precipitate before transfer to second jar of 0.2% gold chloride
27. transfer to a second jar of 0.2% gold chloride for 3 min and continue to move the filters up and down to shake off the precipitate
28. rinse the filters for 10 secs in distilled water & place in 2% oxalic acid for 5 min with gentle agitation
29. rinse well with several distilled water changes over 5 min (when processing several Columbia jars, run each set of filters through steps 23-28 separately to ensure consistent staining)
30. place filters in 5% sodium thiosulfate for 5 min

31. rinse in several changes of distilled water over 5 min
32. dehydrate in isopropyl alcohol series: 30%, 50%, 70%, 90%, 100% #1, & 100% #2 for 5 min each (do not use ethanol or acetone!)
33. soak the filter in xylene, 15 min twice (two separate exchanges)
34. soak the filters in a mixture of 50:50 xylene/permount for at least 1 hour
35. mount the filters; sandwich each filter between 2 drops of permount and cover with a 25 mm square coverslip
36. add permount along the edges of the coverslips to avoid air bubbles & place a weight (e.g. a metal nut and bolt) centered on the coverslip
37. let dry in hood

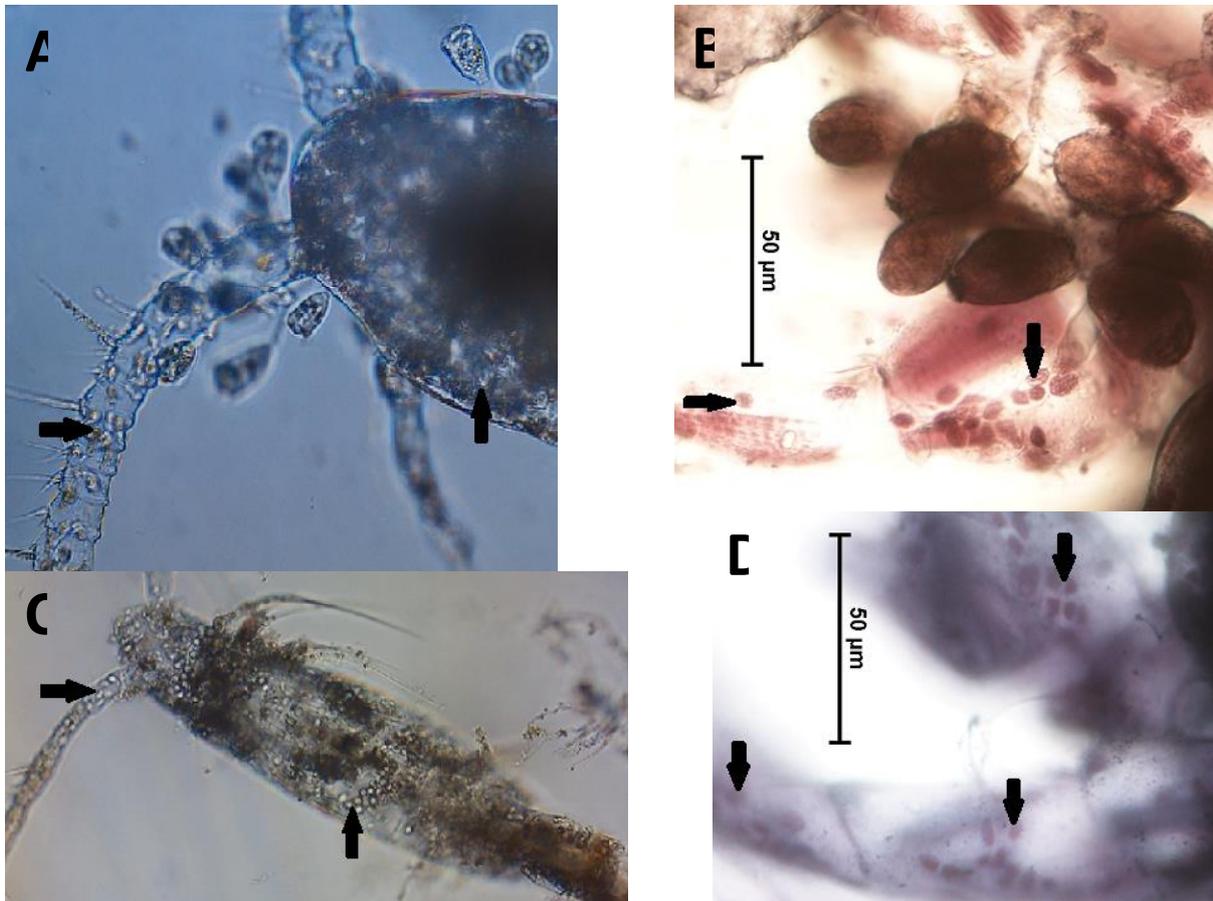
\*To fix samples, apply concentrated Bouin's solution to sample at a final concentration of 5% (v/v). Specimens should remain in fixative for 3-5 days before staining. To make concentrated Bouin's:

- Saturate full strength formaldehyde (37%) with calcium carbonate; this usually takes 3-4 days with periodic shaking
- Decant or filter (Whatmann # 1) calcium carbonate-formaldehyde & saturate with picric acid with constant stirring for at least 24hrs
- Decant or filter calcium carbonate-formaldehyde-picric acid & add glacial acetic acid to 5% (v/v) shortly before use.

\*\* We use only Protargol S<sup>TM</sup>, certified Polysciences

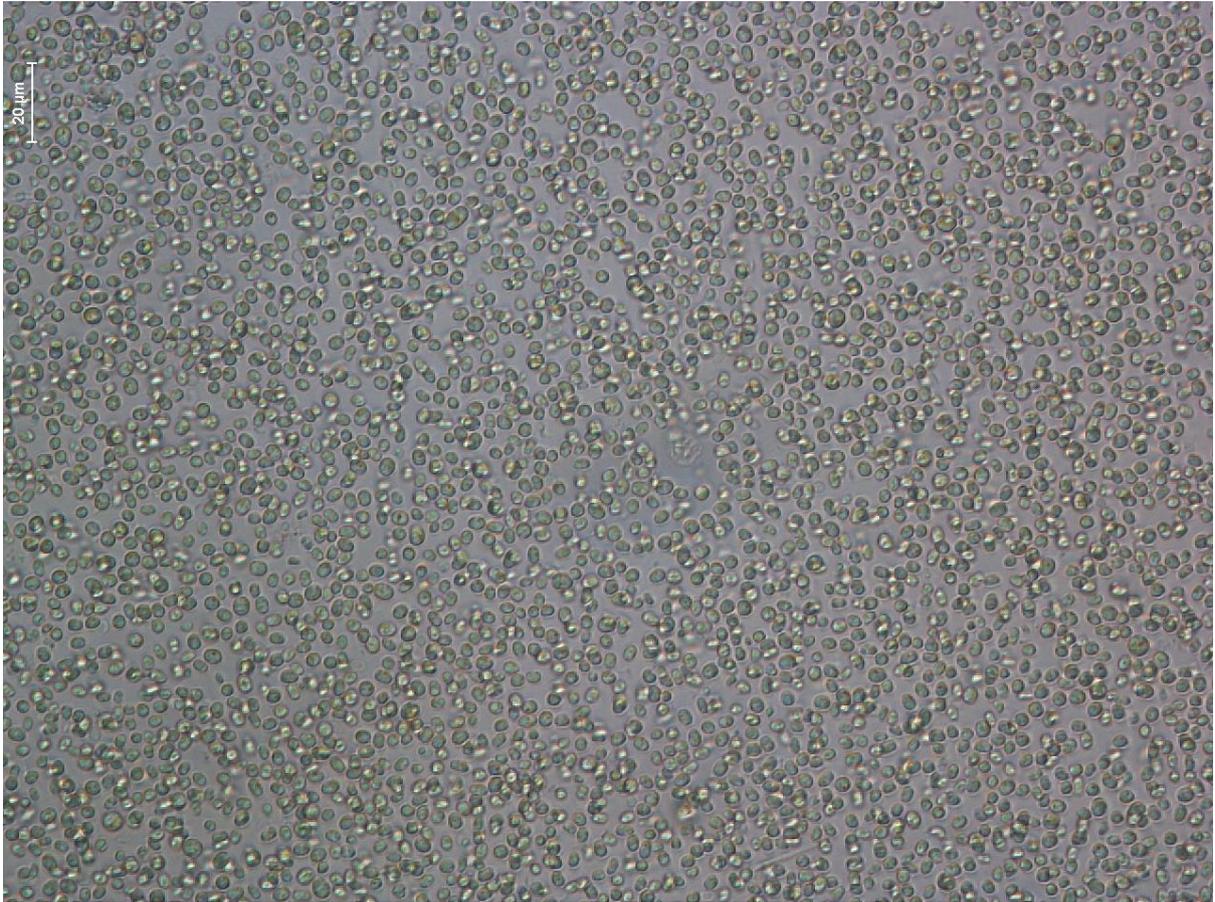


Supplementary material 1.2 A and B: Micrographs showing multiple attempts at using the protargol stain to make the *Epistylis* sp. infraciliature visible.



Supplementary material 1.3: “tomite-like” microorganisms (arrows) swimming inside carcasses of the copepods *Pseudodiaptomus stuhlmanni* (A, B and D) and *Acartiella natalensis* (C). A and C captured these organisms live (March 2014). B and D after protargol-staining of the specimens (September 2017).

## Chapter 5



Supplementary material 5.1: *Chlorophyta* algae used in the temperature-salinity experiments.

**Chapter 6**



Supplementary material 6.1: Micrograph of the unidentified hydrozoan polyp found preying on *Pseudodiaptomus stuhlmanni* in 2016. Note *P. stuhlmanni* inside the gut.



Supplementary material 6.2: Micrograph of the flatworm *Microstomum* sp. found preying on *Pseudodiptomus stuhlmanni* in 2016. Note *P. stuhlmanni* inside the gut.