The potential of algae as indicators of Post Mortem Submersion Interval in a standing body of freshwater

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Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Masters of Science (Medicine) in Forensic Medicine and Pathology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or any examination in any other University.

(Signature of candidate)

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Date

Abstract

While the succession of arthropods is used to determine the *post mortem* interval (PMI) in a terrestrial environment, there is currently no method to determine the *post mortem* submersion interval (PMSI) of a submerged body. Recently, however, the succession of algae colonising submerged remains has received increased attention as a possible PMSI determinant.

The aim of this study was to determine whether algae could be used as PMSI determinants. The decomposition of piglet carcasses were considered. The stages of decomposition and the ADD for each determined. The algae colonizing the substrates (piglet and glass microscope slides) were recorded and any changes in their communities or growth tested to see whether this can be aligned with the submersion period. The effect of seasonality was analyzed using glass microscope slides as changes in the algal community growth and composition would be in response to reasons other than decomposition.

This research was conducted in a freshwater pond in South Africa over three periods (winter 2015, summer 2015 and winter 2018). In 2015 the substrates were submerged for 42 days. The winter study was repeated in 2018 to accommodate complete decomposition. The pH and dissolved oxygen was measured every second day in the winter 2018 study. Samples were obtained every 6 days from a pre-marked surface area of 2,5cm x 2cm along the carcasses' vertebrae and the glass microscope slides. The sampled algae were identified up to the genus level, classified as planktonic (free floating) or benthic (attached to substrate) and a count of each taxon was carried out.

Decompositional changes were documented every two days and five stages of decomposition were determined. The defining characteristics of each stage were present in each study but the duration of the stages differed. The ADD values and stage of decomposition alone were insufficient PMSI indicators. The ADD derived from the TAD scores decreased in accuracy with increasing submersion period. The variation of equations produced in different seasons (current study) and compared with other studies indicates that a new equation will need to be

determined for each scenario. The successful colonisation of the carcasses by algae, the patterns in algal succession and the general bell-shaped curve in algal diversity over time supports the theory that algae, in conjunction with the ADD and degree of decomposition (stages of decomposition), are useful PMSI estimates. The absence of trends between algal diversity and the TAD score prevents the TAD score being used to gauge the degree of decomposition. The bimodal fluctuation of the Shannon-Weiner Index (considers abundance and diversity) is a promising approach in estimating the PMSI but will need to be considered along with the degree of decomposition. The importance of considering season when using algae as PMSI determinants was confirmed by the difference in algal growth and community composition on the glass microscope slides. The variation between seasons and year indicate the need to consider each scenario by itself until such time that the effect of environmental factors can be accounted for. Further research into the effects of seasonal variation and the impact of decomposition on the colonising community could allow ADD, decomposition (TADS and stages) and the algae colonising a submerged body to be used collectively to provide a more informed and complete estimate of the PMSI.

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1 Introduction

Human remains are often found in aquatic systems as a result of homicide, suicide, or accidental causes (Boyle *et al.,* 1996). It is crucial when investigating such cases (Wells and Lamotte, 2010; Dickson *et al.,* 2011; Cockle and Bell, 2015) and to aid in identifying the remains (Seet, 2005; Hardy and Wallace, 2012), that the length of time the body has been submerged in water is established (Evans, 2010; Heaton *et al.,* 2010; Hardy, and Wallace, 2012). In aquatic environments this is termed the *post mortem* submersion interval (PMSI). The PMSI does not denote the period that a body has been deceased but rather the period that it has been immersed. It is possible that the decedent had died prior to being submerged (Heaton *et al.,* 2010; Fink, 2017). Knowing the PMSI however can greatly assist in estimating the total PMI should a portion of decomposition have occurred in a terrestrial environment and aquatic environment (Fink, 2017).

The rate and process of decomposition is affected by chemical, physical and biological factors of the aquatic environment to which the body is exposed (Seet, 2005; Pinheiro, 2006; Parks, 2011; Cockle and Bell, 2015). As the factors acting on a body submerged in water are so different to those on land, the method of colonizing insects used to determine the PMI (*post mortem* interval) on land (Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Vass, 2001; Adlam and Simmons, 2007) cannot be used to determine the PMSI of a submerged body (De Donno *et al.,* 2014; van Daalen *et al.,* 2017). In 2010, Heaton *et al.* modified the scoring method developed for determining the terrestrial PMI (Megyesi *et al.* 2005) to ascertain the PMSI of a submerged body. Although this method has shown promise, the results are unique for each case, as environmental factors are not factored into the prediction model, (Fink *et al.,* 2017). This highlights the need for a method to determine the PMSI of bodies found in

all water systems and to be used for all aquatic cases, including ones in which the body is fully submerged.

Stemming from the successful application of the succession of arthropods colonising decomposing remains on land, the concept of using the successional growth of algae colonising submersed remains should be developed for PMSI estimation (Hardy and Wallace, 2012). The occurrence of algae in all water systems throughout the year allows for this method to be applied to all geographic locations, in all seasons and even to scenarios in which the body has remained submerged during decomposition (Keiper and Casamatta, 2001; Renke, 2010; Hardy and Wallace, 2012).

Throughout the time that the remains are present in the water they provide a substrate to which benthic algae attach and grow. Such algae then establish communities that are subject to change with time (undergo succession). It is thought that the stage in succession of colonising algae can be used to determine the length of time that a body (substrate) has been submerged (Keiper and Casamatta, 2001).

2 Literature Review

2.1 Decomposition

The process of decomposition is a complex one (Vass, 2001; Goff, 2010; Roberts and Dabbs, 2015; Cockle and Bell, 2015) with varying conditions specific to each body, including: the rate (Pinheiro, 2006; Fitzgerald and Oxenham, 2009; Cockle and Bell, 2015), the physical conditions of the body, as well as the specific environment (Pinheiro, 2006; Parks, 2011; Cockle and Bell, 2015).

2.1.1 The internal process of decomposition

The process of decomposition begins approximately four minutes after death (Vass, 2001) and consists of a series of changes that occur once vital activities have stopped (Fitzgerald and Oxenham, 2009). The process of decomposition is carried both by the naturally occurring decomposers inside the body, as well as decomposers in the environment to which the body is exposed. The extent that a corpse is exposed to such decomposers heavily influences the rate at which a body decays (Gunn, 2009).

During decomposition the tissues of the body are broken down primarily by the processes of autolysis and putrefaction (Vass, 2001; Fitzgerald and Oxenham, 2009; Gilbert, 2014; Parks, 2011; Zhou and Byard, 2011).

2.1.1.1 Autolysis

Autolysis, or self-digestion (Zhou and Byard, 2011; Myburgh *et al.,* 2013), is carried out by cellular enzymes released from the cell as the cell membrane loses integrity and bursts. Integrity is lost after death as cells have a decrease in oxygen levels, an increase in carbon dioxide and a decrease in pH (Vass, 2001). Autolytic enzymes begin to break down the tissues of the body (Vass, 2001; Myburgh *et al.,* 2013), with higher autolytic activity occurring in organs with higher levels of enzymes, such as the liver, pancreas, lungs and spleen (Zhou and Byard, 2011). As the body tissues are broken down there is a marked increase in nitrogen compounds and the production of an anaerobic environment (Zhou and Byard, 2011; Myburgh *et al.,* 2013), conditions that favour bacterial growth (Myburgh *et al.,* 2013). Changes associated with the process of autolysis are body cooling (*algor mortis),* pooling of the blood (*livor mortis)* and stiffening of the muscles (*rigor mortis)* (Shkrum and Ramsay, 2007; Myburgh *et al.,* 2013; Zhou and Byard, 2011).

2.1.1.2 Putrefaction

Bacteria also contribute to the decomposition of a body through a process known as putrefaction (Myburgh *et al.,* 2013; Shkrum and Ramsay, 2007; Vass, 2001; Zhou and Byard, 2011). Anaerobic bacteria, such as those of the lungs and intestines (Zhou and Byard, 2011), internally carry out the liquefaction of the soft tissue of the body (Shepherd, 2003; Myburgh *et al.,* 2013) until the body becomes skeletonised (Megyesi *et al.* 2005; Myburgh *et al.,* 2013) Characteristics of early putrefactive stages include abdominal bloating as a result of gas production, emission of odours (Vass, 2001; Myburgh *et al.,* 2013) and green discoloration of the abdominal skin (Vass, 2001). As decomposition advances the bacteria may spread around the body via blood vessels (Goff, 2010; Shepherd, 2003), causing the skin to

have a marbled appearance (Shepherd, 2003; Shkrum and Ramsay, 2007; Goff, 2010; Myburgh *et al.,* 2013).

2.1.2 External decomposers

Microbial decomposers (bacteria, fungi and mould) (Vass, 2001; Pinheiro, 2006; Goff, 2010) that naturally occur on the skin are no longer shed after death and begin to occupy the external surface of the body and contribute to its' decomposition (Goff, 2010). On a larger scale, vertebrate scavengers will often take advantage of the body as a food source and accelerate the rate of decomposition (Vass, 2001; Shepherd, 2003; Pinheiro, 2006; Goff, 2010; Cockle and bell, 2015). The primary decomposers that are known to accelerate decomposition when a body is exposed to air are invertebrates: flies and other arthropods (Vass, 2001; Pinheiro, 2006; Goff, 2010; Cockle and bell, 2015). In aquatic decomposition crustaceans, fish and other small aquatic animals take advantage of the body as a food source and cause a great deal of destruction, particularly to soft parts of the body such as the lips, eyes and eyelids (Petrik *et al.,* 2004).

2.1.3 Products of Decomposition

During decomposition the protein, lipids, carbohydrates and nucleic acids of the body are broken down into simpler units (Swann *et al.,* 2010), such as fatty acids, phenolics (Cobaugh *et al.,* 2015) hydrocarbons, nitrogen compounds, sulphur compounds, acid and ester compounds (Hau *et al*., 2014). These compounds filter back into the surrounding ecosystem (Pechal *et al.,* 2013; Hau *et al.,* 2014) creating a "hot spot" around the remains of enhanced biochemical cycling, including nitrogen cycling, carbon cycling and enhanced phosphodiesterase activity, from which phosphorous compounds are released (Cobaugh *et al.,* 2015). The

release of such compounds has been seen to affect the microbial community structure of terrestrially decomposing carrion with distinct changes in community structure occurring when decomposition fluid is released from the body and after most tissue is decomposed (Pechal *et al.,* 2013; Cobaugh *et al.,* 2015; Finley *et al.,* 2016).

2.1.4 Factors affecting decomposition

The rate at which a body decomposes is extremely variable (Seet, 2005; Pinheiro, 2006) and is dependant on a combination of factors affecting both the external and internal decomposers of the body (Gunn, 2009; Salam *et al.,* 2012; Gilbert, 2014; Keyes *et al.,* 2015). Such factors include temperature (Pinheiro, 2006; Shkrum and Ramsay, 2007; Goff, 2010; Cockle and Bell, 2015), humidity (Pinheiro, 2006; Vass, 2001), physical movement (Pinheiro, 2006) and chemical composition of the environment (Pinheiro, 2006; Gunn, 2009; Cockle and Bell, 2015). While each factor can affect decomposition independently it is usually the combined affect and interrelatedness of these that affect the rate of decomposition (Seet, 2005). Decomposition occurs differently in diverse aquatic environments as the type of water (salt or fresh water), presence of currents and depth of submersion is variable, all of which affect the removal of soft tissue and the process of disarticulation to varying degrees (Haglund, 1993; Seet, 2005).

2.1.4.1 Chemical Factors

The chemical composition of the environment to which a body is exposed has a marked effect on decomposition although very little data is currently available for this (Gunn, 2009). For example, decomposition rates are reduced in salt water as the salt concentration reduces bacteria activity (Boyle *et al.,* 1996; Ayers, 2010; De Donno *et al.,* 2014). Bacteria actions, however, increase in alkaline conditions, resulting in increased rates of putrefaction (Zhou and Byard, 2011).

2.1.4.2 Physical Factors

The unique physical factors of aquatic environments, however such as the presence/absence and strengths of currents, tides and other water turbulences, can increase tissue damage to a submerged body (Haglund and Sorg, 2002; Petrik *et al.,* 2004; Evans, 2010; Heaton *et al.,* 2010; Humphreys *et al.,* 2013;). The exposure of underlying tissues provides easier access for invertebrates (Boyle *et al*., 1996; Gunn, 2009), bacteria (Zhou and Byard, 2011) and other decomposers into the body and leads to an accelerated rate of decomposition (Haglund and Sorg, 2001; Evans, 2010; Heaton *et al.* 2010, Humphreys *et al.,* 2013). Rain has also been found to increase water turbulence (Keiper and Casamatta, 2001; Haefner *et al.,* 2004) and increase the quantities of tissue sloughed from the body (David and Goff, 2000; De Donno *et al.,* 2014). The occurrence of rain can also affect the rate of decomposition negatively by washing away larvae and decreasing the occurrence of insect activity on a floating carcass (Hobischak and Anderson, 2002). Water limits the access of invertebrate decomposers to the carcass (Davis and Goff, 2000) and is regarded as one of the most influential factors in the slow rate of aquatic decomposition (Higgs and Pokines, 2002; Pinheiro, 2006; Gunn, 2009; Evans, 2010).

2.1.4.2.1 Moisture

Water can either increase or decrease the rate of decomposition depending on the quantity, pH and other factors (Haglund and Sorg, 1997; van Daalen *et al*., 2017). The humidity (moisture content) that a body is exposed to whilst decomposing is interconnected to environment temperature and scavenger activity (Seet, 2005). Low environmental humidity increases the rate of carcass desiccation causing it to be an unfavourable environment for scavengers and decreasing the rate that the carrion decomposes (Cockle, 2013). The high humidity of some terrestrial environments near water can cause a delay in the desiccation of a body (Pinheiro, 2006), or in the arrival of insects to that body (Silahuddin *et al.,* 2015) and can even completely exclude scavenger activity (Hobischak and Anderson, 2002). The presence of water in an environment also influences microbial activity, affecting their growth and proliferation. Above or below optimal moisture levels can lead to cessation of microbial activity (Wescott, 2018). Allaire (2002) noted that decomposition and decomposer activity is highly influenced not just by humidity but even more so by temperature.

2.1.4.2.2 Temperature

Temperature and the occurrence of decomposing insects are deemed the most influential variables on decomposition rate (Allaire, 2002), followed by currents and tides (Davis and Goff, 2000, Allaire, 2002). Environmental temperature and that of the body (Shkrum and Ramsay, 2007; Zhou and Byard, 2011) greatly influence the decomposition rate by affecting decomposer activity (Zhou and Byard, 2011). Elevated temperatures lead to an increased rate of autolysis and putrefaction (Shepherd, 2003; Zhou and Byard, 2011), with an increase in odours being emitted from the body causing accelerated manifestation of arthropods and activity of these (Gunn, 2009; Myskowiak *et al*., 2010; Zhou and Byard, 2011).

Temperature extremes, however, decrease decomposer efficiency (Shkrum and Ramsay, 2007; Zhou and Byard, 2011) with temperatures below 10° significantly retarding decomposer activity (Higgs and Pokines, 2002; Zhou and Byard, 2010) and the decomposition process (Zhou and Byard, 2010).

Aquatic decomposition is not subject to harsh temperature fluctuations like terrestrial decomposition as the specific heat capacity of water acts as a buffer, stabilizing changes in the water temperature (King, 1996). The rate of decomposition is decreased in lower water temperatures (Evans, 2010; Petrik *et al.,* 2004) with the resurfacing time of a body taking longer in situations where the body is in cooler and deeper waters (Haglund and Sorg, 2001; Myskowiak *et al*., 2010).

2.2 Stages of Decomposition

2.2.1 Terrestrial and Floating Aquatic Decomposition

The process of decomposition is commonly grouped into stages (Pinheiro, 2006; Adlam and Simmons, 2007; Gennard, 2007) for both terrestrial and aquatic decomposition. The number and distinction of these varies between studies and authors, as do the names of the stages (Pinheiro, 2006; Adlam and Simmons, 2007; Gennard, 2007; Goff, 2010; Keyes *et al.,* 2015). This results in decomposition being classified into as few as three stages (Weigelt,1927) or as many as 21 stages (Adlam and Simmons, 2007; Fitzgerald and Oxenham, 2009). For the most part decomposition is classified into five distinct stages for terrestrial decomposition. Floating aquatic decomposition was initially classified into

six stages of decomposition (Payne and King, 1972). In 2000, Davis and Goff identified five stages of floating decomposition (given in Table 1 below) each with the general characteristics of their counterparts in terrestrial decomposition (Table 1).

Table 1: Decompositional characteristics of each of the five stages of decomposition occurring in a terrestrial environments and an aquatic environment where the body is allowed to float to the water surface.

	Terrestrial	Floating Aquatic
Stage 1	Fresh Stage	Submerged Fresh Stage
	Greenish discolouration of the abdomen Skin cracking Tache noir (discolouration of the eyes) Insect invasion at body openings (Goff, 2010)	Negatively buoyant No insects present (Seet, 2010) Gases begin to fill abdomen Flies occurring on part exposed to air (Davis and Goff, 2000) Body skin firm (Petrik *et al.,* 2004)
Stage 2	Bloated Stage	Early Floating Stage
	Abdomen has bloated appearance Maggots present at primary invasion sites Seeping of fluids from body (Goff, 2010)	Positively buoyant Bloated appearance Colonised by insects (Haglund and Sorg, 2001; Haefner *et al.,* 2004) Bubbles emerge from nose, mouth and anus (Seet, 2010) Odour present (Davis and Goff, 2000) Exposed portion of abdomen becomes discoloured (Seet, 2010)
Stage 3	Decay Stage	Floating Decay Stage

	Decreased bloated appearance due to rupturing of skin of abdomen Strong odour Large maggot masses (Goff, 2010)	Maggot masses present (Alley, 2007; Seet, 2010) Beetles can colonise the body (Seet, 2010) Reduction in tissue as skin detached from body (Davis and Goff, 2000; Alley, 2007) Exposure of bone on body parts that have a thin layer of skin / tissue covering the bone (Haglund, 1993) Oil film present in water surrounding the body (Davis and Goff, 2000)
Stage 4	Postdecay Stage	Bloated Deterioration Stage
	Body is reduced to skin, cartilage and bone Diptera (flies) are no longer the predominant feature Presence of Coleoptera (beetles) (Goff, 2010)	Body begins to sink Increased maggot masses and dead maggots in the water (Davis and Goff, 2000; Alley, 2007) Majority of soft tissue consumes Froth present on carcass and in water (Davis and Goff, 2000; Alley, 2007; Seet, 2010)
Stage 5	Skeletal Stage	Sunken Remains Stage
	Only bones and hair remain No definite end point to this stage (Goff, 2010)	Carcass is reduced to dried skin and bone (Alley, 2007; Seet, 2010) or fat and bones (Davis and Goff, 2000) Negatively buoyant (David and Goff, 2000; Alley, 2007; Seet, 2010) Bacteria and fungi present in the water complete the process of decomposition (Alley, 2007; Seet, 2010)

2.2.2 Fully Submerged Decomposition

In some situations a body might be deliberately or accidentally unable to float to the surface and in these circumstances the remains will then stay submerged throughout the decomposition process (Lawler, 1992). There are five stages for submerged decomposition, namely Submerged Fresh, Early Floating, Floating Decay, Advanced Floating Decay and Sunken Remains (Haefner *et al.,* 2004; Zimmerman and Wallace, 2008).

2.2.2.1 Stage 1 – Submerged Fresh Stage

The carcass still has a fresh appearance with no obvious signs of decomposition present (Haefner *et al*., 2004; Dickson *et al*. 2011) The carcass sinks in the water (Haefner *et al.,* 2004; Zimmerman and Wallace, 2008).

2.2.2.2 Stage 2 – Bloated Stage

During the second stage of decomposition the carcass becomes positively buoyant, because of gases produced by anaerobic gut biota, and has a bloated appearance. The change in buoyancy causes the carcass to press up into any obstructing object, often causing indentations of this onto the skin of the carcass (Haefner *et al.,* 2004; Zimmerman and Wallace, 2008). The skin has a marked increase in flaccidity (Dickson *et al.,* 2011) with visible algal growth occurring in freshwater (Haefner *et al.,* 2004) and brackish water habitats (Zimmerman and Wallace, 2008). In a marine environment it was said that there was a thin layer of slime covering the submerged analogues and an odour present (Anderson and Hobischak, 2002; Dickson *et al.,* 2011).

2.2.2.3 Stage 3 – Floating Decay Stage

Minor decay occurs in the third stage with skin sloughing from the carcass and a notable decrease in muscle mass (Haefner *et al.,* 2004; Zimmerman and Wallace, 2008). Eyes and soft tissue detach and the legs and head of the piglet analogue used remain intact (Haefner *et al.,* 2004) allowing the carcass to be still identifiable as a piglet (Haefner *et al.,* 2004; Zimmerman and Wallace, 2008). Dickson *et al*. (2011) noted the presence of a strong odour during this stage.

2.2.2.4 Stage 4 – Advanced Floating Decay Stage

Tissue deterioration increases markedly during the fourth stage (Haefner *et al.* 2004; Zimmerman and Wallace, 2008) with high levels of skin sloughing (Dickson *et al.,* 2011) leading to the exposure of the ribs and skull (Haefner *et al*., 2004; Dickson *et al.,* 2011). Throughout this stage, bones begin to disarticulate and the bones of the skull begin to separate (Haefner *et al.,* 2004; Dickson *et al.,* 2011; Zimmerman and Wallace, 2008) causing an increased difficulty in identification of the analogue (being that of a piglet) (Haefner *et al.,* 2004; Zimmerman and Wallace, 2008).

2.2.2.5 Stage 5 – Sunken Remains Stage

The final stage of decomposition begins once the remains are reduced to skin and bones. Research in which the analogue was restrained in a cage described the remaining skin and tissue liquid to be decomposed to where they were that of a thick liquid (Haefner *et al.,* 2004; Zimmerman and Wallace, 2008). Dickson *et al.* (2011) characterised the final stage of decomposition as that when no identifiable soft tissue remained and all

odour was lost. This stage continues as the remaining bones are slowly broken down with there being no set completion of this (Haefner *et al.* 2004).

2.3 Pigs as Human Analogues

Pigs are frequently used as analogues for humans in research involving entomology and taphonomy due to the similarities in tissue, muscular structure and the process of decomposition (Goff, 1993; Matuszewski *et al.*, 2019). The question as to whether pigs serve as an accurate proxy for a human body was addressed by Matuszewski *et al.* (2019). They noted that, despite pig analogues having been invaluable in understanding the process and patterns of decomposition, there are differences between the decomposition of pigs and humans. Dissimilar body proportions, gastrointestinal anatomy, diet (Matuszewski *et al*., 2019) and loads of external and internal bacteria (Keough *et al*., 2017) contribute to such differences. Matuszewski *et al.* (2019) recommended that larger pigs (>30kg) be used to provide more accurate data which can be applied to scenarios involving human bodies, especially when it comes to studies considering the later stages of decomposition.

The use of piglets as analogues provides data that is most relatable to newborn babies (Renke, 2010) or even a child (Haglund and Sorg, 2010). This is because of the comparably smaller size, and therefore less flesh to decompose, and the greater surface-to-volume ratio of the piglets (Haglung and Sorg, 2001). The composition of the gut microbiota also contributes to the similarity between the piglet and a newborn baby (Renke, 2010). Nonetheless, both pigs and piglets serve well in “proof-of-concept” studies of the decomposition of humans (Matuszewski *et al*., 2019).

2.4 Determination of the Post Mortem Interval (PMI) and Post Mortem Submersion Interval (PMSI)

The characteristic changes that occur to a body are used to determine the PMI or PMSI of a body (Gennard, 2006; Pinheiro, 2006; Goff, 2010; Zhou and Byard, 2011). Even though the PMI and PMSI may not be equivalent, (Fink, 2017), the changes that occur in a decomposing body do not occur at a steady and predictable rate (Gennard, 2006; Pinheiro, 2006; Goff, 2010). This is driven by the individual fluctuation and combined effect of factors affecting decomposition, such as temperature and wind (in the terrestrial environment; Goff, 2010), and water flow and depth (in the aquatic environment; Haglund, 1993), and they can change during the decomposition process. The changes to a decomposing body immediately after death occur at a faster rate than those associated with the later stages of decomposition (Goff, 2010). The irregularities of these make the determination of the PMI of a body on land and PMSI of a body in water challenging (Gennard, 2006; Goff, 2010; Pinheiro, 2006) with a decrease in accuracy the longer the body is submerged.

2.4.1 ADD (Accumulated Degree-Day) Method

The ADD method is based on the concept that in order for a particular amount of decomposition to occur to a body the body must be exposed to a correlating amount of energy. This energy is usually that of thermal energy obtained from the environment in which the body is decomposing (Simmons *et al.,* 2010; Myburgh *et al.,* 2013; De Donno, 2014). The interrelatedness of temperature, degree of decomposition and period of submersion allow for the PMSI to be determined through the ADD method and, because temperature affects decomposition in all environments, allows a comparison between locations (Heaton *et al.,* 2010; De Donno *et*

al., 2014). The differing abiotic factors and analogue type impacts decomposition and causes the ADD to differ between studies (Table 2). Zimmerman and Wallace (2008) found that water movements also significantly contribute to the rate of decomposition for aquatic environments. Strong currents (Haglund and Sorg, 2001; Humphreys *et al.,* 2013), tidal fluctuations and turbulence provide constant movement and shear stress on the surface of a body causing an increase of tissue sloughing from the bones (Haglund and Sorg, 2001) and accelerate the rate of decomposition (Humphreys *et al.,* 2013).

Table 2: ADD per stage of decomposition of aquatic studies with differing water type, season and analogue type.

	Haefner *et al.* (2004)		Dickson *et al.* (2011)	Zimmerman and Wallace (2008)	
Analogue Type	Pig		Pig heads	Piglet	
Season	Winter		Winter	Spring	Summer
	Freshwater stream	Freshwater pond	Marine	Brackish	Brackish
Stage 1 Fresh Stage	76.8	151.2	40	70.86	51.33
Stage 2 Bloated Stage	118.1	88.6	28	161.97	152.62
Stage 3 Floating Decay Stage	70	152.3	20	117.27	90.47
Stage 4 Advanced Floating Decay Stage	64.9	242	53	208.4	130.77
Stage 5 Sunken Remains Stage	34	139.2	-	-	130.77

2.4.2 Visual Scoring Method (VSM)

A problem in using the stages of decomposition to estimate the PMSI and PMI is the subjective and qualitative nature of observations of soft tissue decay (Keyes *et al.,* 2016). The objective, quantitative point-based scoring system developed by Megyesi *et al.* (2005) overcomes such downfalls. Megyesi *et al.* (2005) performed a human decomposition rate study in the USA for terrestrial environments. In the method different areas of the body were individually assessed and assigned a score according to the stage of decomposition of the area. Decomposition of the head, neck and cervical vertebrae was used to determine the facial decomposition score (FDS); decomposition of the limbs, hands and feet to determine the limbs decompositional score (LDS) and the torso, pelvis, pectoral girdle and abdomen to determine the body decompositional score (BDS). The decomposition processes of each body region was divided into quantified stages, each of which were assigned point values. As the different body regions do not experience the same decomposition changes (e.g. limbs do not purge decomposition fluids), each body region was scored independently. A higher total body score (TBS) was associated with more advanced decomposition with the maximum TBS score possible of 25. The lowest possible score was 3, commensurate with fresh decomposition in all three regions of the body. These authors considered such a quantitative approach to classifying degree of decomposition more objective and thus a better estimate of PMI (Megyesi *et al.,* 2005).

An advantage of using a scoring system is that it can be applied to both human and animal analogues. The degree of decomposition can be determined as the scoring is based on decompositional changes and scored body regions that occur on both analogues (Myburgh *et al*., 2013; Matuszewski *et al.,* 2014; Keyes *et al*., 2016). In addition, the TAD score can be determined at the scene of recovery with no autopsy required. The TADS method is therefore less time consuming (Reijnen *et al*., 2018).

Another advantage of the TBS is that inter-observer error between independent data capturers (even those with different experience) is considerably reduced to the point of insignificance (Dabbs, Connor & Bytheway 2016).

The process and rate of decomposition in water is determined by the environment to which the body is exposed and the condition of the body itself (Haglund and Sorg, 2001; Evans, 2010). In an attempt to increase the accuracy of PMSI estimates of human remains in UK waterways, Heaton *et al.* (2010) used the visual scoring method previously devised by Megyesi *et al.* (2005), for terrestrial systems to determine the PMSI of a body in an aquatic environment (Heaton *et al.,* 2010; Humphreys *et al.,* 2013). To determine the degree of decomposition of the carcass the total aquatic decomposition (TAD) score was calculated. The TAD score is obtained by summing the facial aquatic decomposition (FAD) points, the body aquatic decomposition (BAD) points and the limb aquatic decomposition (LAD) points (Heaton *et al.,* 2010; Humphreys *et al.,* 2013). The TAD score is then plotted against the calculated ADD and it is this relationship of degree of decomposition to time and temperature that was used to calculate an approximate PMSI (Heaton *et al.,* 2010; Humphreys *et al.,* 2013; van Daalen *et al.,* 2017).

Humphreys *et al.* (2013) calculated the PMSI of piglet carcasses submerged in a manmade water reservoir using the TADS scoring system. The resulting equation of this prediction model differed to that determined by the method founder, Heaton *et al.* (2010). The difference was attributed to a difference in methods (location and analogue type). A problem encountered in research by Humphreys *et al.* (2013), who attempted to monitor the algal growth on the piglet carcasses and the TADS *in situ,* was that it was difficult to note the physical changes of the carcass through the algae colonising it whilst the carcass remained submerged.

In 2017, van Daalen *et al.* (2017) reported that the TADS method proved promising in estimating the PMSI but additional information relating to the trajectory of the body should also be considered to allow for a more accurate PMSI estimation. Drifting of a body further from the coastline or a change in depth of submergence of the body would affect the temperature to which it was exposed. The importance of considering additional factors that affect decomposition of a submerged body was also mentioned in research by de Donno *et al.* (2014). Failing to do so would result in a false sense of accuracy in the method of TADS in estimating the PMSI (de Donno *et al.*, 2014).

Although the visual scoring method has been successful when applied to PMI estimates of a terrestrial setting, the conditions to which a body is exposed to in an aquatic setting cannot be factored in making it unreliable for an aquatic setting (Dickson *et al.,* 2011; Humphreys *et al.,* 2013; De Donno *et al.,* 2014).

2.4.3 Forensic Entomology – colonising arthropods

A well-researched and reliable method for PMI determination is the use of the sequential and somewhat predictable colonisation of the body by specific terrestrial insect species (Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Shepherd, 2003; Pinheiro, 2006; Gennard, 2007; Gunn, 2009; Kaliszan *et al.,* 2009; Wells and Lamotte, 2010; Cockle and Bell, 2015; Matuszewski *et al.,* 2015). Terrestrial insects have been less successfully used for aquatic decomposition (Tomberlin and Adler, 1998; Chin *et al.,* 2008) as the carcass is only floating and exposed to terrestrial insects for a portion of decomposition and the length of exposure is dependant on the rate of decomposition (MacDonell and Anderson, 1997; Hobischak and Anderson, 2002;) The duration of exposure varies with water depth, proportion of body floating above the water line, size of the body, water flow, temperature and the presence of aquatic flora and fauna

(MacDonell and Anderson, 1997; Hobischak and Anderson, 2002). The location and the occurrence of the terrestrial insects, that would take advantage of the body as a food source, in this location also affects whether this approach can be used to determine the PMSI (Chin *et al.,* 2008). The degree of colonisation is reduced because water prevents colonisation of the submerged portions of the body (Keiper and Casamatta, 2001; Petrik *et al.,* 2004; Chin et al., 2008;). Hobischak and Anderson, (2002), found that the high humidity and low temperature associated with most aquatic environments caused the absence of maggot masses on the floating carcass bringing into question whether terrestrial insects can successfully be used for PMSI determination.

2.4.4 Aquatic Insects

With the shortfall in the use of terrestrial insects in aquatic settings research was conducted to determine whether aquatic insects could be as successful in PMSI estimation as their terrestrial counterparts (MacDonell and Anderson, 1997; Tomberlin and Adler, 1998; Hobischak and Anderson, 2002).

Aquatic insects feed on algae, decaying plant matter, and other insects (Haskell *et al.,* 1989) accounting for the occurrence of these on a carcass onto which the sought after food has settled or attached. No taxa exclusively feed on a submerged carcass as aquatic insects have not evolved adequate feeding mechanisms and physiological adaptions to allow this, straining the potential use of these in PMSI determination (Haskell *et al.,* 1989; Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Haefner *et al.,* 2004; Zimmerman and Wallace, 2008). It has, however, been found that midges and caddisflies are capable of colonising an immersed carcass, and with the understanding of the life cycle and biology of these species, can be used to determine the PMSI. The

application of this method, however, is limited to geographic locations in which these species occur (Haskell *et al.,* 1989).

2.5 Colonisation of submerged substrates

The colonisation of a submerged substrate by microorganisms has been documented using various substrates but has only recently been applied to forensic cases (Hobischak and Anderson, 2002; Benbow *et al*., 2015; Lang *et al.,* 2016).

Colonisation of a substrate will only be accomplished if the substrate type and environmental conditions meet the needs of the colonising taxa (Benbow *et al.,* 2015; Lang *et al.,* 2016). The surface of substrates must be sufficiently suited for the attachment abilities of colonising taxa and large enough to accommodate these. Community structure may also be affected by the shape of the substrate, with grooves and channels providing protection from predators or shielding colonisers from harsh water flow (Hobischak and Anderson, 2002). The community structure is altered by the nutrients release from the substrate itself and those present in the environment in which it is submersed (Benbow *et al.,* 2015; Lang *et al.,* 2016).

2.5.1 Change in Diversity of Colonising Organisms

In general, there is a bell-shaped curve of diversity (species richness) over time (successional period) (Figure 1). At first diversity increases as the substrate is colonised by pioneering species that have characteristics that enable them to rapidly colonise a substrate. With increasing time other colonising species occur and the maximum level of diversity is reached when pioneering species, mid-successional species and late colonisers, referred to as climax species in Townsend *et al*. (2008), are all present on

the substrate, (Biggs and Smith, 2002; Townsend *et al.,* 2008). If there is no disturbance, diversity then decreases as stronger competitors (late colonisers) establish and displace many of the earlier colonisers (Townsend *et al.,* 2008).

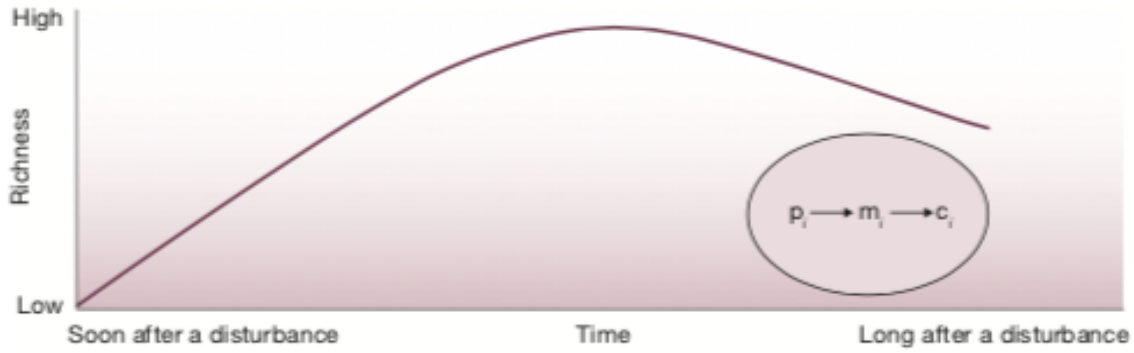


Figure 1: Hypothetical succession of uncolonized surface of a dominance controlled community. Richness starts at a low level as a few pioneer (p_i) (early coloniser) species arrive. It reaches a maximum in mid-succession when a mixture of pioneer, mid-successional (m_i) and climax (c*i*) (late coloniser) species occur together and decreases as competitive exclusion by late colonisers (c_i) species occurs (After Townsend *et al*., (2008), pg 301).

2.5.2 Biofilm Community and PMSI

Once submersed, a biofilm layer forms on the substrate surface and is comprised of bacteria, fungi, protozoa and algae, encased in an extracellular polymeric substance (Benbow *et al.,* 2016; Lang *et al.,* 2016). Bacteria are the first colonisers and remain the base layer for subsequent establishment of larger microorganisms (Benbow *et al.,* 2015; Lang *et al.,* 2015). With increasing period of submersion larger and filamentous algae and fungi dominate the biofilm (Benbow *et al*., 2015) and, if a suitable food source is available, lead to the attraction of aquatic invertebrates (Myskowiak *et al.,* 2010). It is this successional change over in colonising organisms with time that is thought to be useful in determining the PMSI of submersed carrion (Benbow *et al.,* 2015; Lang *et al.,* 2016).

The succession of bacteria colonising piglet carrion submersed in freshwater streams was researched using pyrosequencing, a method using DNA to allow the identification of bacterial taxa (Benbow *et al.,* 2015), and ribosomal intergenic spacer analysis (ARISA), which creates and compares the unique fingerprint of the communities using the 16S-23S intergenic space in bacteria (Lang *et al*., 2016). Analysis of the biofilm bacteria has also been conducted in a marine setting using capillary electrophoresis sequencing and, unlike studies conducted in freshwater environments, used adult pig heads (Dickson *et al.,* 2011). Community composition and succession were different between seasons (Dickson *et al.,* 2011; Benbow *et al*., 2015), locations, and even at different depth gradients of the same location (Lang *et al.,* 2016), all of which highlight the influence of environmental factors on colonising community composition and succession. Even with such differences, the successional change of the community over time is hypothesised to be useful in determining the PMSI (Dickson *et al.,* 2011; Benbow *et al.,* 2015; Lang *et al.,* 2016).

The biofilm community colonising a submerged carcass is not only made up of bacteria, but includes fungi and algae (Benbow *et al.,* 2015; Lang *et al*., 2016). To our knowledge no research has been conducted on fungal communities on a submersed carcass and limited research has been conducted on algal colonisation of a submerged carcass and the application of this in PMSI determination (Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Haefner *et al.,* 2004; Zimmerman and Wallace, 2008).

2.5.3 Disturbance Ecology

To use the algal community to determine the PMSI of decomposing remains one would need to consider how decomposition of the carcass affects the colonising algal community. Disturbance ecology is a theory that a moderate increase in disturbance leads to an increase in diversity of

the colonising algae, as it displaces dominating taxa and affords opportunity for the establishment of others. However, extreme disturbance again depresses diversity as intolerant taxa are lost and only a few resilient and better-suited taxa persist (Biggs and Smith, 2002; Townsend *et al.,* 2008).

If algae are to be used as a method to determine the PMSI of a body, it is important to consider that changes in the colonising algae (chlorophyll a concentration, biomass, diversity and community composition) will not just be a function of time. As the body undergoes decomposition, the periods of increased disturbance will increase the amount of tissue being sloughed from the body (Adlam and Simmons), and thus decrease the amount of attached algae (Casamatta and Verb, 2000; Renke, 2010).

2.4 Algae and Forensics

The involvement of algae and forensics is not a new concept as diatoms (*Bacillariophyta*), a specific taxonomic division of unicellular algae (Casamatta and Verb, 2000; Horton *et al.,* 2006; Zimmerman and Wallace, 2008), have been used to confirm death as a result of drowning if found in the victim's lungs (Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Horton *et al.,* 2006; Lunetta *et al.,* 2014). This indicates that the victim inhaled these algae simultaneously with water, a finding that would not occur if the victim was already dead before submersion (Keiper and Casamatta, 2001; Lunetta *et al.,* 2014). The location of a drowning event has also been determined by comparing the algal assemblage found in the victim with those of various aquatic sites (Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Zimmerman and Wallace, 2008; Lunetta *et al.,* 2014). The ability of diatoms to withstand harsh environmental conditions allows them to be used in forensic cases after death has occurred and decomposition ensues (Keiper and Casamatta, 2001; Zimmerman and Wallace, 2008). The presence of particular algae species

on items of clothing and elsewhere has also been used to link criminals to a crime scene (Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Haefner *et al.,* 2004; Coyle *et al.,* 2005; Zimmerman and Wallace, 2008;) and to link components of a crime (Keiper and Casamatta, 2001; Coyle *et al.,* 2005).

The occurrence of algae in all seasons (Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Zimmerman and Wallace, 2008; Hardy and Wallace, 2012) and in all water habitats makes it an appealing tool in forensics (Keiper and Casamatta, 2001; Horton *et al.,* 2006), furthered by the ease of identification without the need for high-end machinery (Keiper and Casamatta, 2001; Stevenson *et al.,* 2011).

2.4.1 Algae

Algae are a phylogenically diverse group of photosynthesising organisms that occur in a wide range of aquatic habitats (Stevenson *et al.,* 2011) and are important primary producers (Lange *et al.,* 2011). Some species are free floating in the water whereas others are benthic and occur on substrata submersed in water (Stevenson *et al.,* 2011). For a substrate to be colonised by a particular algal species there must be sufficient resources that are needed, such as nutrients, adequate light and space (Stevenson *et al.,* 2011). Growth and composition of the colonising community is also shaped by the degree of physical disruption of the substrate, temperature of the water and the occurrence of herbivores (Lange *et al.,* 2011). The collective influences of these shape the algal community causing the community to be dominated by species that are best suited to the environment, as is the case for low level of light and nutrient environments having a large amount of low profile benthic species as they can gain the required amount of nutrients from the substrate they are in close relation to (Lange *et al.,* 2011).

The first colonisers of a substrate are adnate algae, usually motile diatoms, as their close relation to the substrate surface and ability to secrete mucilage allow for an easy attachment to the substrate (Sekar *et al.,* 2004). Their close relation to the substrate offers protection from the shearing stress of currents (Stevenson *et al.,* 2011). With increasing submersion periods of the substrate stalked algae occur followed by filamentous algae, both of which are non-motile and can create nutrient and light limiting conditions for first colonisers (Stevenson *et al.,* 2011). Motile forms, however, can move to more suitable conditions overcoming such limitations (Lange *et al.* 2011; Stevenson *et al.* 2011).

The different requirements of each algal taxon causes a change in the algal community structure over time (succession) (Stevenson *et al.,* 2011) and it is this succession that is hoped to provide a timeline from which the length of time the substrate (body) has been submersed can be determined (Keiper and Casamatta, 2001).

2.4.2 Algae and PMSI

The change in the colonising benthic algae community structure whist the carcass is submersed is thought to mimic the successional change of arthropods colonising a carcass in terrestrial habitats and be useful in tracing back the length of time a body has been present in water (Keiper and Casamatta, 2001; Hardy and Wallace, 2012).

The first to investigate the use of algae for PMSI was Casamatta and Verb (2000). Rat carcasses were submerged in freshwater streams and the colonising algae analysed over a period of 21 days. Diatoms were the first to colonise the rat carcasses. With increasing submersion time more complex algae, such as those of the phylum (or division) *Chlorophyta* (Green algae), colonise the carcass increasing algal diversity. The slower growth rate of these taxa is proposed to be the reason for these taxa

appearing later in submersion, with carcasses requiring more time to be conditioned by these (Casamatta and Verb, 2000; Biggs and Smith, 2002). Casamatta and Verb (2000) found the number of diatom epiphytes, algae that grows on larger filamentous species, a possible indicator of the PMSI as there will be fewer epiphytes present on a carcass recently submerged than one submerged for a longer period of time and that is colonised by slower growing filamentous algae. Casamatta and Verb (2000) suggested that decomposition of the carcass caused the habitat to become more acidic.
In the slow flow pool habitat this favoured an increase in desmids, a division of Chlorophyta, whereas the flow of the riffle habitat prevented a build-up of acidic conditions, accounting for the delay of desmids on the submersed rat carcasses in this area (Casamatta and Verb, 2000).

Haefner *et al.* (2004) focused their attention on whether the total growth of benthic algae colonising a submerged carcass could be a quantitative indicator of the PMSI. Through analysis of the chlorophyll-a concentration they found a quantitative link between algal growth and the period of submersion from which the authors believed the PMSI could be determined. They also highlighted factors that could affect this relationship, including rainfall and quantity of nutrients released from the decomposing carcass, and recommended that these be considered when using this approach.

In 2008, Zimmerman and Wallace produced a semi-quantitative approach using algal diversity to determine the PMSI. The piglet carcasses were submerged in brackish water and the algal species colonising these were compared to the stages of decomposition of the carcasses. The algae colonising ceramic tiles was also analysed and the results found ceramic tiles inadequate to simulate mammalian carcasses. With increasing period of submersion, and advancing decomposition of the submerged piglet carcasses, fewer types of algae were observed. Zimmerman and Wallace,

(2008), attributed this to high levels of nutrients released during decomposition and disarticulation of the body decreasing the available surface area for algal colonisation.

Renke (2010) conducted the most recent research using algae to determine the PMSI. Similar to the findings of Haefner *et al*. in 2004, Renke (2010) observed that chlorophyll-a concentration of the algae colonising the piglet increased with the submersion period of the piglet. Unlike other studies, however, the piglet carcasses were unrestricted and floated to the water surface during the study. Renke (2010) considered the effect of season on the application of algae as a PMSI estimate. The increased rate of decomposition and algal growth in the warmer season highlights the need to consider season when using algae to estimate the PMSI. Despite the potential of rainfall events to negatively impact algal growth through increased shearing forces on decaying tissue used as a substrate by the algae, chlorophyll- a concentration was found to increase with submersion period for all substrates and was seen as a possible method to determine the PMSI.

Renke (2010) was the first to address the difference of seasons on chlorophyll concentration and PMSI estimation focusing on temperature differences. In both the research by Haefner *et al*. (2004) and Renke, (2010) rain events were found to dislodge the algae attached to the carcass, increasing tissue loss from the submerged carcass and resulted in a decrease in algae. It was cautioned that such conditions should be considered when using algae to determine the PMSI. Seasonal differences that can influence the colonising algal community include light levels (Coutinho and Seeliger, 1986; Cantonati and Lowe, 2014), temperature, intra- and interspecific competition (Stewart and Lowe, 2008) and nutrient availability (Coutinho and Seeliger, 1986). As a result of such seasonal variations, the benthic algal community differs across seasons (Coutinho and Seeliger, 1986; Keiper and Casamatta, 2001; Haefner *et*

al., 2004; Hardy and Wallace, 2012; Lang *et al.,* 2016). It stands to reason that such variations also would impact any community colonising a submerged carcass in different seasons.

2.5 Conclusion

The estimation of PMSI is crucial in investigations of human deaths when the body is recovered from water. This study investigated whether the algae colonising submerged remains can be used to determine the PMSI.

While the potential of algae in forensic science has been investigated in various locations in America (Casamatta and Verb, 2000; Haefner *et al.,* 2004; Zimmerman and Wallace, 2008; Renke, 2010) no such studies have been undertaken in South Africa.

3 Aim and Objectives

3.1 Aim

The aim of this study was to determine whether algae could potentially be used to estimate the PMSI of a submerged piglet carcass in Johannesburg, South Africa.

3.2 Objectives

The objectives of this study were:

1. To document the process of decomposition of a submerged piglet carcass to distinguish and describe each of the stages of decomposition and determine the duration and ADD of these in summer and winter.
2. To determine whether algae can colonise a carcass throughout decomposition and, if so, whether changes in their communities or growth can be aligned with the period of submersion.
3. To determine the difference of algal succession on artificial substrates (glass microscope slides) across seasons. The inclusion of the glass microscope slides in this study allows one to determine whether seasonality impacts on algal growth and affects its potential as a PMSI indicator. Including the change of the algal community on glass microscope slides allows one to determine whether changes in algal community structure on the piglet are due to reasons other than decomposition.

4 Methods and Materials

This is a quantitative, longitudinal, prospective study.

4.1 Ethics

Ethics approval was granted by the Animal Ethics Screening Committee (AESC). Clearance certificate number: 2014/63/O (Appendix 1).

4.2 Period of Study

The study was conducted over three periods covering two seasons. The winter 2015 study ran from 6 June 2015 to 17 July 2015, the summer from 26 September 2015 to 6 November 2015 and the winter 2018 study from 1 May 2018 to 4 September 2018. Initially only the winter and summer 2015 studies were planned so that the impact of sequential seasons on decomposition could be unpacked. A study period of 42 days study period was set aside for each study. When the winter 2015 study was terminated after the assigned 42 day study period the carcass had yet to complete the decomposition process. It was then decided that, in order to have a complete data set for the entire process of decomposition in both seasons, an additional winter study (2018) had to be undertaken to follow the entire process of decomposition.

4.3 Study Site

The study was conducted in a man-made freshwater pond in the residential suburb of Northcliff, Johannesburg, South Africa (GPS coordinates -26° 9' 8.2836"S 27° 56' 54.8052"E). The terrain surrounding the pond perimeter has a low profile so the pond is exposed to direct sunlight through most of the day. The water of the pond was not changed between the studies.

The volume of the pond was estimated at 5325 litres. The surface area was calculated by superimposing a grid with a mesh size of 0,5 metres by 0,5 metres over the surface of the pond. The depth of the water in each square element of the grids was measured and the volume of these determined and summed. Volume estimates of incomplete peripheral squares were added to this sum. The dimensions of the pond and the distance of the submerged carcass and glass microscope slides were measured (Figure 2).

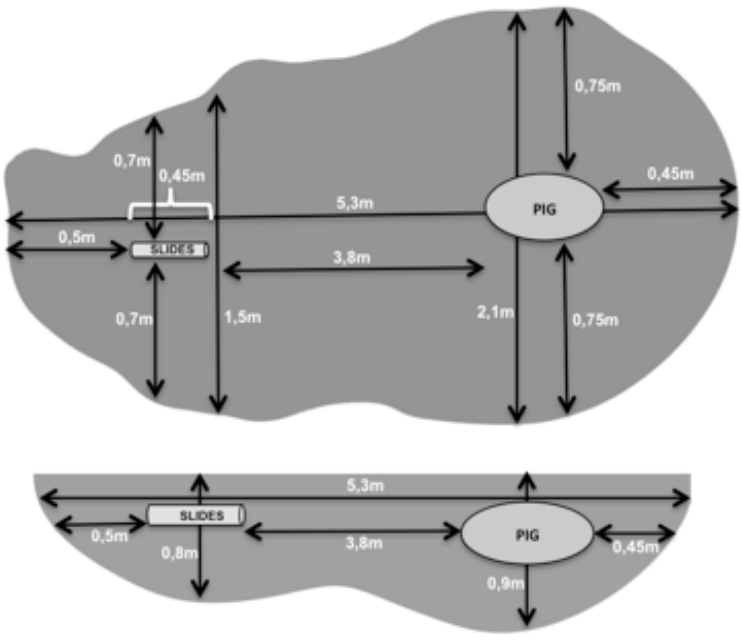


Figure 2: Dimensions of pond and submerged substrates.

4.4 Substrates

Three domestic piglets (*Sus scrofa domesticus)* of similar size (see section 4.4.1) were used in this study because they have been widely accepted as proxies of human corpses when documenting the process of decomposition (MacDonell and Anderson, 1997; Goff, 1993).

A comparison of the algal community on the glass microscope slides was carried out in summer and winter in 2015 to determine whether a seasonal variation existed in the background communities of the pond. A direct comparison between the algae colonising the glass microscope slides and the piglet carcass was avoided as each colonising community will differ because of the differing substratal structure and chemistry (Keiper and Casamatta, 2014; Schneck and Melo, 2011). The general trends in succession (Townsend *et al.,* 2008) would however occur on both of the substrates. The difference would be that changes in the algal community on the artificial substrate would not be affected by the process or products of decomposition whereas those colonising the piglet carcass substrate would be. Differences noted in the dynamics of the algal community on the slides between seasons, or indeed years, highlights any environmental variation excluding the impact of decomposition, as would be experienced by those communities growing on the piglet. The glass microscope slides and piglet were submerged simultaneously in the pond.

4.4.1 Collection and Preparation of Piglet Carcass

The piglets used in this study were stillborn and were obtained from Topigs SA's Dorstfontein branch (a pig breeding farm) (GPS Coordinates 25°58'39.05"S, 28°36'50.47"E). All three of the piglets used in the study died due to natural causes approximately 48 hours prior to receipt, had been refrigerated in the interim and had no notable external injuries. At commencement of the study, the piglet used in the winter 2015 study

weighed 875g, that of the summer study 902g and the piglet of the winter 2018 study weighed 897g.

The carcasses were transported to the study site in a cool, dry polystyrene box. In order to retard decomposition, the piglets were kept cool throughout transportation using frozen ice packs. To avoid tissue damage the piglets were kept separate from the packs by placing them in a smaller polystyrene box placed inside that containing the packs.

The piglet used for the winter 2015 study still had its umbilical cord attached and the amniotic sac intact. Prior to being submerged, the amniotic sac of this piglet was removed with a dry paper towel. The umbilical cord was left in place to avoid the creation of any open wound site. Before being placed into the water, all three carcasses were wiped down with a dry paper towel, in order to remove remnants of dirt and mucus on the skin, and weighed using a digital scale. Along the spine a line of 12 (winter and summer 2015) and 21 (winter 2018) blocks of 2cm x 2,5cm was marked, from which samples were taken (Figure 3). As decomposition advances an increasing amount of tissue is sloughed from the body. The sample site along the vertebral column of the piglet was chosen as it is one of the last areas where tissue detaches and bones disarticulate during decomposition (Haglund, 1993). The sample site was still close enough to be subjected to the effects of decomposition, in particular the abdominal rupturing.

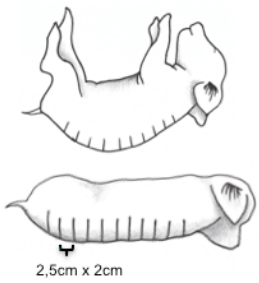


Figure 3: Sample site marking made on the back of the piglet carcasses, measuring 2cm X 2.5cm.

4.5 Experiment Set Up

4.5.1 Piglet

Each piglet was placed on its right side (left side facing upward) on a metal grid (Metal grid 2, Fig. 4) that in turn was supported by two columns of three bricks each Another grid (Metal grid 1, Fig. 4), supported by a single brick on either side of the pig and secured at a depth of about 25 cm, was secured by further bricks to prevent the carcass from floating to the surface or from being removed by opportunistic scavengers (Figure 4). During the winter 2015 study the restraining metal grid (1) was made of chicken mesh but this was replaced by a thicker and more robust stainless steel grid. This was done because the grid in the winter 2015 study was found to flex to the point of exposing the carcass to the air on day 18 of the study. Fortunately, the exposure was limited to approximately five minutes as it occurred during the collection of the algal samples. As exposure was for approximately 5 minutes, the effect that this had on the study was deemed negligible as this was the approximate length of time the carcass was exposed to air when algal samples were collected on sample days. To prevent exposure of the piglet to the air, the grid and bricks had to be frequently adjusted taking care not to disturb the piglet.

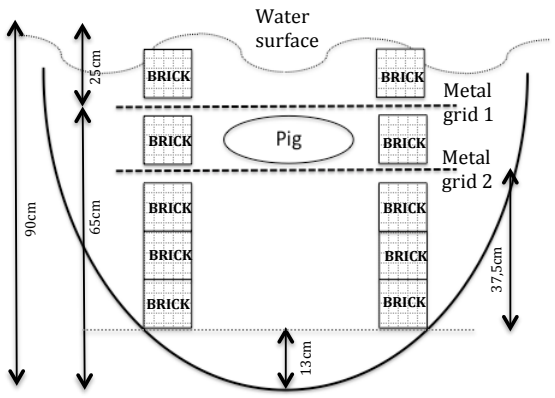


Figure 4: Experimental set-up designed used to keep the carcass submerged during both studies.

During the summer study the pond water was topped up with roughly 50 litres of tap water on the eighteenth and thirty-second day of the study to ensure that the water level remained within the pre-marked water level. This ensured that the top of the piglet remained at a depth of 20cm to 25cm below the surface. In the winter months, evaporation was negligible.

4.5.2 Artificial Substrates

Glass microscope slides were thoroughly cleaned with a dry paper towel and a 2cm by 2.5 cm area marked off using a permanent marker pen. Slits were cut into a solid polystyrene cylinder (7cm in diameter) with 2,5cm spacing between each slit. A single glass microscope slide was then inserted into each slit, ensuring that each 2cm x 2,5cm sample area was fully exposed. Five additional slides were included to account for any contingencies. The polystyrene tube was horizontally submerged into the pond and anchored at each end by a thick string tied to 2 bricks (Figure 5). The tube was positioned to have the glass slides at the same depth (20 to 25 cm), with the same aspect (North-facing) and orientation (vertical) as the sampling area of the carcass.

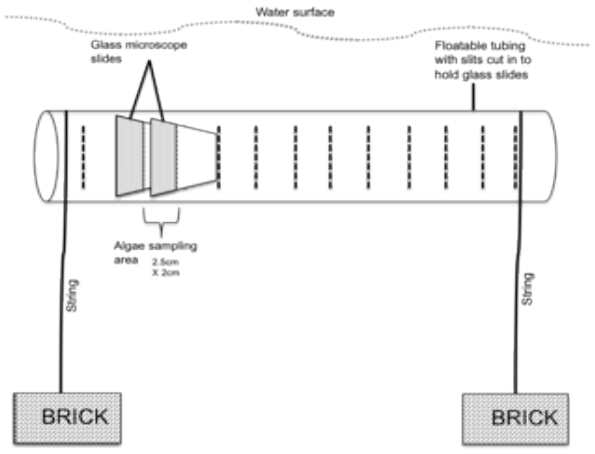


Figure 5: Design of polystyrene tubing holding the glass microscope slides.

4.6 Temperature and ADD

The water temperature was measured hourly using a Thermocron® iButton® (Model: DS1922L) placed 25 cm below the water surface, the same depth as the piglet carcass. Prior to submersion, the iButton was dipped in melted bee's wax (about 3mm thick) to waterproof it. This thin coat was deemed to have negligible effect on temperature transfer to the metallic iButton (a good conductor of temperature) that is completely immersed in water. In addition, water is an effective buffer of temperature change and so changes would never be marked. The hourly recorded data, downloaded once at the end of each study, were converted to average daily temperatures and totalled to determine the ADD for each completed stage of decomposition.

4.7 Dissolved oxygen and pH

The pH and dissolved oxygen were measured every day in the winter 2018 study using a YSI (Yellow Springs Instrument) probe. The probe was submerged in the water before any sampling or photographic documentation was done so as to avoid disturbance of the water and the effect that such unavoidable mixing the water around the carcass would have. The probe was submerged to the same depth as the carcass and recordings were taken approximately 10 cm away from the vertebral side of the carcass. Recordings were taken on the vertebral side of the carcass as this was where the samples were being collected from the carcass. The results were recorded on the data sheet.

The pH and dissolved oxygen were only recorded in the winter study 2018 study as it was only recognised after the data of the 2015 studies were collected that such environmental variables may have significant bearing on the response of the algae to decomposition.

4.8 Rainfall

A rainfall report was obtained from the South African Weather Bureau Services for the nearest weather station (GPS coordinates Lat: 26°09'22"S, Lon: 27°59'56"E) for September to November 2015 (Appendix 2, 3 and 4) . These data were used to determine whether rainfall impacted on algal growth. As no rain occurred in the winter 2015 and winter 2018 studies the rainfall reports for these periods were not requested.

4.9 Documentation of Decomposition Process and Determining the Stage of Decomposition

On every second day any decompositional changes on the carcass were recorded on a data collection sheet (Appendix 5). The physical changes were annotated and sketches on the piglet diagrams on the data collection sheet. This required the removal of the upper grid (1) and of the piglet with the support of grid 2 from the water for a maximum of 5 minutes. Prior to the removal of the covering metal grid (1), notable changes to the carcass and surrounding water were noted.

The defining characteristics of each of the five stages of decomposition used in this study were taken from the research of Haefner *et al.* (2004) and Zimmerman and Wallace (2008). Those referenced by Dickson *et al.* (2011) were of limited value as the authors utilised submerged body parts of pigs and not whole pigs. Based on the physical decompositional changes the carcass was classified into one of the five stages proposed by Haefner *et al.* (2004).

The carcass was also removed from the metal grid and placed on a neutral background (polystyrene board) for photography to facilitate documentation of the decomposition process. However, for the winter

2015 study, photographs were taken whilst the carcass remained submerged. Afterwards it was returned and submerged in the water. Photographs corresponding to stage of decomposition were compared across seasons.

4.9.1 Total Aquatic Decomposition Score (TADS)

The stage of decay of each carcass was scored using a semi-quantitative evaluation of decomposition. Each carcass was classified based on post-mortem changes according to the decomposition scoring system designed by Heaton *et al*. (2010) (Table 3). The Total Aquatic Decomposition Score was determined using observations made when the carcasses were removed from the pond. The scores for the Facial Aquatic Decomposition Score and Torso Aquatic Decomposition Score range from 1 to 8 and up to 9 for the Limb Aquatic Decompositional Score. The summation of these produced the Total Aquatic Decompositional Score (TADS). By determining the TADS, and knowing the daily water temperatures, the ADD determined from a derived linear equation was used to backtrack and calculate the PMSI.

Table 3: Descriptive stages of decomposition observed on the head/neck, torso and limbs and assigned decompositional scores. Modified from Heaton *et al.* 2010.

Score	
1	Head/neck: no visible or relevant changes. Trunk: no visible or relevant changes. Limbs: no visible or relevant changes.
2	Head/neck: slight pink discoloration, darkened lips, goose pimpling. Trunk: slight pink discoloration, goose pimpling. Limbs: mild wrinkling of skin on hands and/or feet, possible goose pimpling.
3	Head/neck: reddening of face/neck. Marbling visible on face/neck. Possible early signs of animal activity/predation concentrated on the nose, ears, and lips. Trunk: yellow/green discoloration of the abdomen and upper chest. Marbling. Early decomposition/autolysis of internal organs. Limbs: skin on palms of hands and/or soles of feet becoming white, wrinkled, and thickened. Slight pink discoloration of arms and legs.
4	Head/neck: bloating of the face. Green/black discoloration. Skin beginning to slough off. Trunk: dark/green discoloration of abdomen, mild abdominal bloating. Initial skin slippage. Limbs: skin on palms of hands and/or soles of feet becoming soggy and loose. Marbling of the limbs predominantly on upper arms and legs.
5	Head/neck: head hair beginning to slough off mostly at the front. Brain softening and becoming liquefied. Tissue becoming exposed on face and neck. Green/black discoloration. Trunk: green/purple discoloration, extensive abdominal bloating, tense to touch, swollen scrotum in males, exposure of underlying fat and tissues. Limbs: skin on hands/feet starting to slough off. Yellow/green to green/black discoloration on arms and/or legs. Initial skin slippage on arms and/or legs.
6	Head/neck: bone becoming exposed, concentrated over the orbital, frontal, and parietal regions. Some on the mandible and maxilla. Early adipocere formation. Trunk: black discoloration, bloating becoming softer, initial exposure of internal organs and bones. Limbs: degloving of hands and/or feet — exposing large areas of underlying muscles and tendons. Patchy sloughing of skin on arms and/or legs.
7	Head/neck: more extensive skeletonization on the cranium. Disarticulation of the mandible. Trunk: further loss of tissues and organs, more bone exposed, initial adipocere formation. Limbs: exposure of bones of hands and/or feet. Muscle, tendons, and small areas of bone exposed in lower and/or legs.
8	Head/neck: complete disarticulation of the skull from torso. Extensive adipocere formation. Trunk: complete skeletonization and disarticulation. Limbs: bones of hands and/or feet beginning to disarticulate. Bones of upper arms and/or legs becoming exposed.
9	Limbs: complete skeletonization and disarticulation of limbs.

4.9.2 Analysis of Decomposition Data

The daily average temperature was calculated at the end of each study period and the used to determine the ADD for each stage of decomposition. The ADD was re-expressed as $\log_{10}$ ADD based on the recommendations made by Simmons *et al.* (2010) and De Donno *et al.* (2014) who found that decompositional scoring systems plotted against $\log_{10}$ADD allows for the expression of a simple linear equation that represents the exponential progression of decomposition. The correlation between the TAD score, and $\log_{10}$ ADD produced an equation that can be used to calculate the ADD of a body should the environmental temperature to which the body was exposed be known. The calculated ADD and the actual ADD were plotted against the TAD scores every six days of submersion for all three studies. Comparing the calculated and actual ADD allows one to determine whether determining the PMSI from the derived equation would be accurate throughout the submersion period or if accuracy would decrease with increasing submersion period.

4.10 Algal Sampling

Algae were sampled every six days over a 42 day period for two 2015 studies, and over a 132 day period for the winter 2018 study. The restraining metal grid was removed, allowing the carcass to float to the surface during sampling. Samples were taken from the scheduled pre-marked sample area of the carcass and from one of the glass microscope slides. A sample was taken from the carcass by scraping the skin within the pre-marked borders of the sample area with a clean dry scalpel blade and then rinsing it off in 25ml of distilled water in a hard plastic sample container with a plastic screw-on lid. The algae were similarly scraped from the marked area of the slide after the latter was carefully removed from the holder.

The sample jars were labelled with the substrate type and collection date. Thereafter they were stored in a dry plastic cooler box until identification later the same day.

4.10.1 Analysis of Algal Data

The sampled algae were identified to genus level using dichotomous keys of Prescott (1978) and van Vuuren *et al.* (2006). The colonizing species were further classified as either planktonic (species that do not attach to the substrate and include Diatoms) or benthic (species that do attach to the substrate). The number of individuals of each species from a known aliquot of the sample was determined and converted to a count for the entire sample, representing the number for the specific surface area scraped. Algal diversity and abundance per sample day was determined for all substrates in all of the three studies.

The number of each algal taxon colonizing each substrate was converted into a percentage of the total number of colonizers per substrate for each sample day. Taxa which accounted for less than 2 % of the total colonizing community were grouped under “other”.

The Sorenson’s Similarity Index (C_s) was used to determine whether the composition of the algal taxa on both the piglet and the glass microscope slides in summer 2015 were similar to those in winter 2018. Each sample from each substrate type and each sample period was directly compared with its counterpart in the other season. This approach occluded any differences in the composition of the colonising community that was the result of a difference in substrate type.

A Sorenson's value of 1,0 indicates a perfect match of species between the two communities, whereas 0,0 indicates complete dissimilarity. The equation used is as follows:

$C_s = 2C/(S1+S2)$

Where, C_s = Sorenson's Similarity Index; C= the number of species the two communities have in common; S1= the number of species in the first community; S2= the number of species in the second community.

While Sorenson's Similarity Index only considered the number of taxa present in each sample, the Shannon Weiner Equitability Index (H) considers both the abundance and the distribution (evenness) of these. The Shannon Wiener Equitability Index was determined only for the summer 2015 and winter 2018 studies, as only these covered the complete decompositional process. This allowed for changes in diversity over the submersion period to be considered with the influence of decomposition present in one data group (piglet) and not the other (glass microscope slides). The index was determined for every directly comparable sample. The Shannon Wiener Equitability Index ranges from 0 to 1, where 1 indicates an even frequency of the species of a community. Algal diversity for each sample date (six day submersion interval) was determined using the Shannon-Weiner Index. To facilitate comparison, the period of submersion when each sample was collected was represented as a percentage of the total submersion period.

The formula for Shannon-Weiner Index is as follows:

$H = - [\sum Pi (lnPi)]$

Where, H = Shannon Weiner Diversity Index; Pi = the proportion of each species in the sample; lnPi = natural logarithm of this proportion.

Algal diversity (benthic and planktonic) was plotted against the TAD score for all three studies to determine whether there was a trend in algal diversity relative to the TADS (derived from the degree of decomposition). If TADS were used to denote the degree of decomposition of the carcass, and the algal growth used to determine the PMSI of it, the trends in algal growth need to be considered against the TADS.

A regressional analysis of ADD, Shannon- Weiner Diversity Index and stage of decomposition was carried out for all seasons. The days of submersion (time) was the independent variable in this analysis and the p-value used was 0,05. The Shannon-Weiner Diversity Index of the piglet carcass was plotted against the change in algal diversity, pH, dissolved oxygen and water temperature over the submersion period. This was done to determine whether the pH or dissolved oxygen was brought on by decomposition of the piglet carcasses or rainfall events and whether changes in these impact algal diversity and abundance.

The data analyses were completed through Microsoft Excel 2018 (Microsoft Corporation, Redmond, WA).

5 Results

5.1 Decomposition Analysis

5.1.1 Stage 1 – Submerged Fresh Stage (Figure 7a-c)

At the start of all of the studies the piglets were fresh and had been dead for approximately 24 hours. There was no discoloration or any other obvious signs of decomposition on any of the carcasses. When placed into the water all of the carcasses were negatively buoyant.

Green discolouring of abdominal skin (Figure 6) began after two days of submersion in the summer 2015 study, after eight days in the winter 2015 and after fifteen days in winter 2018. As decomposition continued the piglets had more of a bloated appearance. Despite the minor bloating, as all of the carcasses remained negatively buoyant, they were regarded as being in the first stage of decomposition.

Some algae were observed to have settled onto the upper surface of all of the carcasses but were readily dislodged when the carcasses were disturbed, perhaps causing colonisation to take longer than would have otherwise been the case.

5.1.2 Stage 2 – Bloated Stage (Fig. 7d-f)

During the second stage, the abdomen of all of the piglets became visibly distended with gases and, as decomposition progressed, the bloating extended into the upper parts of the limbs. The gases produced during decomposition caused positive buoyancy on the fifth day of submersion in the summer study, day nine in the winter 2015 and day 22 in the winter 2018 study. This positive buoyancy caused indentations on the upper surface of the carcasses as they pressed into the restraining grid.

In both seasons, all of the carcasses had abdominal discolouration in the second stage of decomposition, ranging from light to dark purple-grey (Figure 6). This discolouration spread from the abdomen to the upper hind limbs, and did so more rapidly in the warmer summer water.

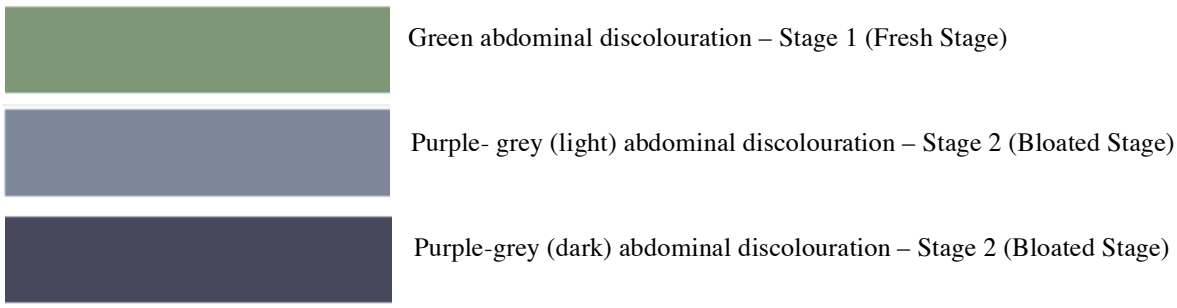


Figure 6: Colour swatches relevant to the characterisation of abdominal discolouration during the Bloated Stage of decomposition (After www.materials-world.com).

The tissue of the carcasses began to soften and slough off, with the rear left trotter completely detaching during the summer study and winter 2018 study. The increased pressure in the abdomen caused gas bubbles to emanate from the buccal cavity in all studies. In addition, a partial extrusion of the hindgut occurred in the winter 2015 and winter 2018 subjects.

During this stage, a noticeable slime film was present on the surface of all three carcasses but was more obvious in the summer study. This slime layer was, for the most part, clear and had a slight green colouration. As decomposition progressed a layer of green algae became noticeable on the surface of the summer carcass. It was estimated that 20 – 30% of the algae covering the top surface of the piglet, excluding previously sampled areas, was adhering to the carcass. The winter 2015 and 2018 carcasses had a less obvious layer of green algae on its surface. When disturbed the algae in the winter 2015 and 2018 studies would detach indicating that the piglets had not been significantly colonised by benthic algae at this time.

5.1.3 Stage 3 – Decay Stage (Figure 7g-h)

The start of the third stage was marked by the rupturing of the abdominal wall with a concomitant release of abdominal gas, body fluids and decomposing organs into the surrounding water. As a result, there was a notable decrease in bulk of all of the carcasses, which remained positively buoyant.

The winter 2015 study reached the six-week termination mark a day after the Decay stage (third stage) had commenced. The study was deliberately terminated as the submersion period for the 2015 studies was set at 42 days. After the winter 2015 study ended on day 42 observations were restricted to the summer study.

The end of the third stage was marked by obvious sloughing of the tissue and skin from the lower limbs and jaw, partially exposing the underlying bone and tendons. Nevertheless, both the carcasses still retained sufficient cohesiveness to be recognisable as piglets.

There was an increase in the algae present on the carcass at the start of the third stage in both the summer 2015 and winter 2018 studies and the

algae developed further as decomposition progressed. When tissue, and the algae attached to it, was sloughed off new uncolonised surface areas were exposed and were accessible for potential colonisation. It was estimated that roughly 60 – 70% of the algae present on the unsloughed skin on the superior surface of the piglet remained attached when the carcass was lifted out and then returned to the water.

5.1.4 Stage 4 – Advanced Floating Decay Stage (Figure 7i-j)

During this stage there was an increase in the incidence of tissue loss with the bones and parts of the tendons of the upper leg becoming exposed. The bones of both the upper and lower jaw were clearly visible and, at the end of the fourth stage (day 34, summer 2015; day 135, winter 2018), the lower jaw disarticulated in both the summer 2015 (day 36) and winter 2018 (day 131) studies, and the cranium fell apart in the summer 2015 study (day 40).

The extensive tissue loss resulted in the loss of an estimated 50 – 60% of the algae that had established on the carcass. On areas where the tissue remained, such as parts of the rib cage and pelvis, the colonising algae continued to increase in density and produce a thick green algal mat. Parts of the piglet carcasses where tissue detached or bones disarticulated had a thinner algal covering causing the carcass to have a patchy appearance.

5.1.5 Stage 5 – Sunken Remains (Figure 7k-l)

The fifth stage was marked by bones largely uncovered by decomposing flesh, with the rib cage clearly visible, and now with the carcass having negative buoyancy.

Thick algal growth was on the remaining skin on the spinal column and ribs, whereas areas where tissue had detached had varying degrees of algal attachment as previously uncolonised surface areas were exposed and then colonised at different rates.

A summary of the identifiable characteristics and duration of the five stages of decomposition identified in this study is given in Table 4 below. The duration of the final stage of decomposition (Sunken Remains) is indeterminate as the bones continue to degrade with time.

Table 4: Summary of descriptions and duration of the five stages of decomposition identified in this study during summer 2015 and winter 2018 where decomposition of the piglet carcass was completed.

Stage of Decomposition	**Summer 2015**	**Winter 2018**
Stage 1 Fresh Stage	Duration: 4 days	Duration: 21 days
	Description	
	• No discolouration / obvious signs of decomposition to a greenish discolouration evident on the abdomen • Negatively buoyant • Bloated appearance increases throughout the stage	
Stage 2 Bloated Stage	Duration: 19 days	Duration: 41 days
	Description	
	• Abdomen is visibly distended • Bloated appearance extends to the upper portion of the limbs • Gas bubbles emanate from the buccal cavity and hindgut extrudes • Positively buoyant • Abdominal discolouration from green to purple, to a purple-grey • Abdominal discolouration extends to the	

<table>
<tr><td></td><td colspan="2">upper hind limbs
• Tissues soften and begin to slough off
• Trotters begin to detach
• Slime present on skin
• Settling of algae on the top (upward facing) surface of the carcass</td></tr>
<tr><td rowspan="3">Stage 3
Decay Stage</td><td>Duration: 10 days</td><td>Duration: 41 days</td></tr>
<tr><td colspan="2">Description</td></tr>
<tr><td colspan="2">• Abdominal wall ruptured
• Body fluids and decomposing organs in the surrounding water
• Associated decrease in the bulk of the carcass
• Sloughing of tissue from lower limbs and jaw
• Partial exposure of bone and tendon on lower limbs and jaw
• Carcass still recognisable as that of a piglet
• Colonising algal turf patchy as sloughing tissue exposes fresh bare areas</td></tr>
<tr><td rowspan="3">Stage 4
Advanced Floating Decay Stage</td><td>Duration: 8 days</td><td>Duration 28: days</td></tr>
<tr><td colspan="2">Description</td></tr>
<tr><td colspan="2">• Increase in tissue loss
• Bones and tendons of upper limbs visible
• Bones of upper and lower jaw visible
• Disarticulation of lower jaw and, in the latter parts of the stage, of the cranium
• Dense alga turfs in areas where sloughing absent (pelvis and rib cage)</td></tr>
<tr><td rowspan="3">Stage 5
Sunken Remains Stage</td><td>Duration: indefinite</td><td>Duration: indefinite</td></tr>
<tr><td colspan="2">Description</td></tr>
<tr><td colspan="2">• Majority of the carcass only present as exposed bones
• Rib cage clearly visible
• Negatively buoyant
• Thick algal growth evident on what skin remains (spinal column and ribs)
• Varying degrees of algal attachment evident on remaining tissue and bones</td></tr>
</table>

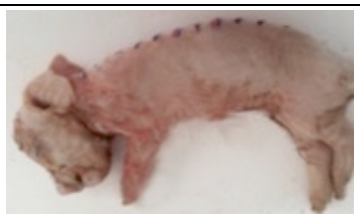
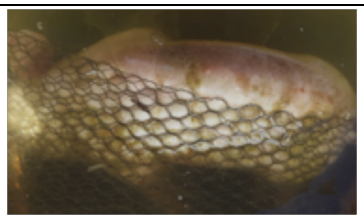
Stage of Decomposition	Winter 2015	Summer 2015	Winter 2018
Stage 1 Floating Fresh Stage	 Day 1 – 8 Captured on day 1	 Day 1 – 4 Captured on day 1	 Day 1- 21 Captured on day 6
Stage 2 Bloated Stage	 Day 9 – 42 Captured on day 36	 Day 5 -23 Captured on day 12	 Day 22 – 62 Captured on day 30
Stage 3 Floating Decay Stage		 Day 24 – 33 Captured on day 24	 Day 63 – 103 Captured on day 66
Stage 4 Advanced Floating Decay Stage		 Day 34 – 41 Captured on day 36	 Day 104 – 131 Captured on day 120
Stage 5 Sunken Remains Stage		 Day 42 Captured on day 42	 Day 132 Captured on day 132

Figure 7: Photographic documentation and duration of each stage of decomposition of the piglet for each of the three studies.

5.2 Total Aquatic Decomposition Score (TAD Score)

The steeper slope in the summer 2015 study indicated there was a greater increase in decomposition relative to the increasing log_{10}ADD in the summer 2015 than the other studies. Comparison of the two winter studies indicated that the TAD score was lower for log_{10}ADD in winter 2015 than winter 2018. The relationship (slope) of log_{10}ADD and TADS score was more comparable for summer 2015 and winter 2018 studies than the two winter studies (Figure 8).

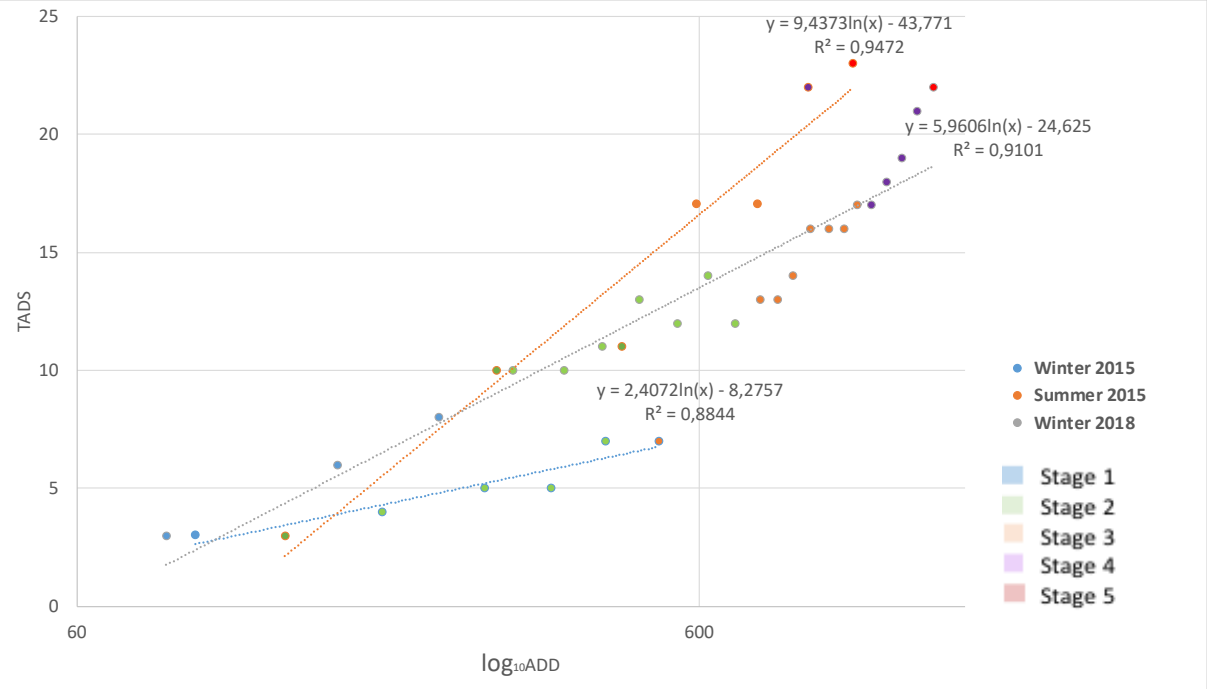


Figure 8: Total Aquatic Decompositional Score (TADS), as calculated from Heaton *et al*. (2010) plotted against log_{10}ADD. The corresponding stage of decomposition of the studies are represented by the colour of the data point markers and as shown in the key on the right of the graph.

A plot of the actual ADD and calculated ADD vs TADS (Figure 9) showed that with increasing TADS (decomposition), the difference between the calculated ADD and the actual ADD increased in all of the studies. Therefore, as decomposition increases the ADD calculated from the equation becomes less accurate. The most notable difference occurred on the last sample date of the winter 2018 study (day 132) where the ADD calculated from the derived equation was 2495,4 ADD when the actual ADD at this time was 1428,3 ADD.

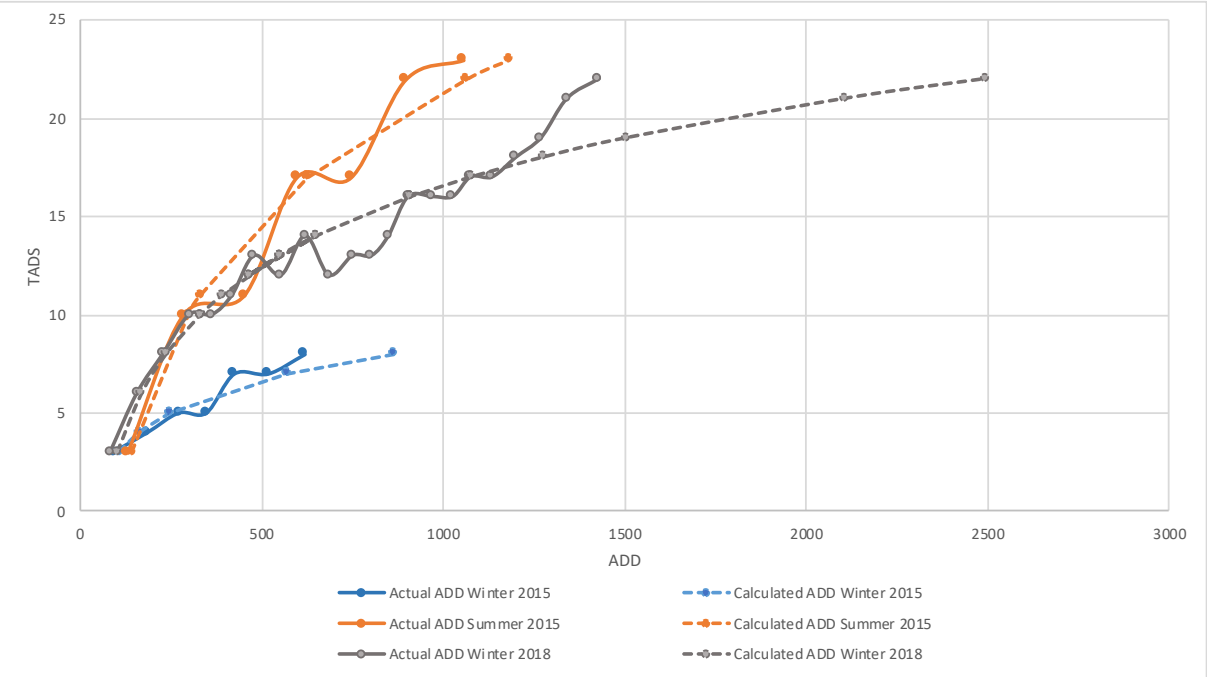


Figure 9: The actual ADD and the calculated ADD (from the relevant linear correlation equation per study) are plotted against the TAD score.

5.3 Temperature, Accumulated Degree Days (ADD), Decomposition and Rainfall

The only rainfall events in the study occurred on day 38 (26.4 mm) and day 39 (27.2 mm) of the summer 2015 study. The lowest daily average temperatures experienced for the season also occurred on these days of the summer 2015 study ($\bar{x}$ = 19.50 °C, sd= 4.50 °C and $\bar{x}$ = 19.13 °C, sd= 4.05 °C respectively), but temperature was consistently higher than in either of the winter studies (Fig. 10). The water in winter 2018 experienced the coldest temperature ($\bar{x}$ =10,9 °C, sd= 1,87 °C) and resulted in the slowest rate of decomposition of all the studies (Figure 10).

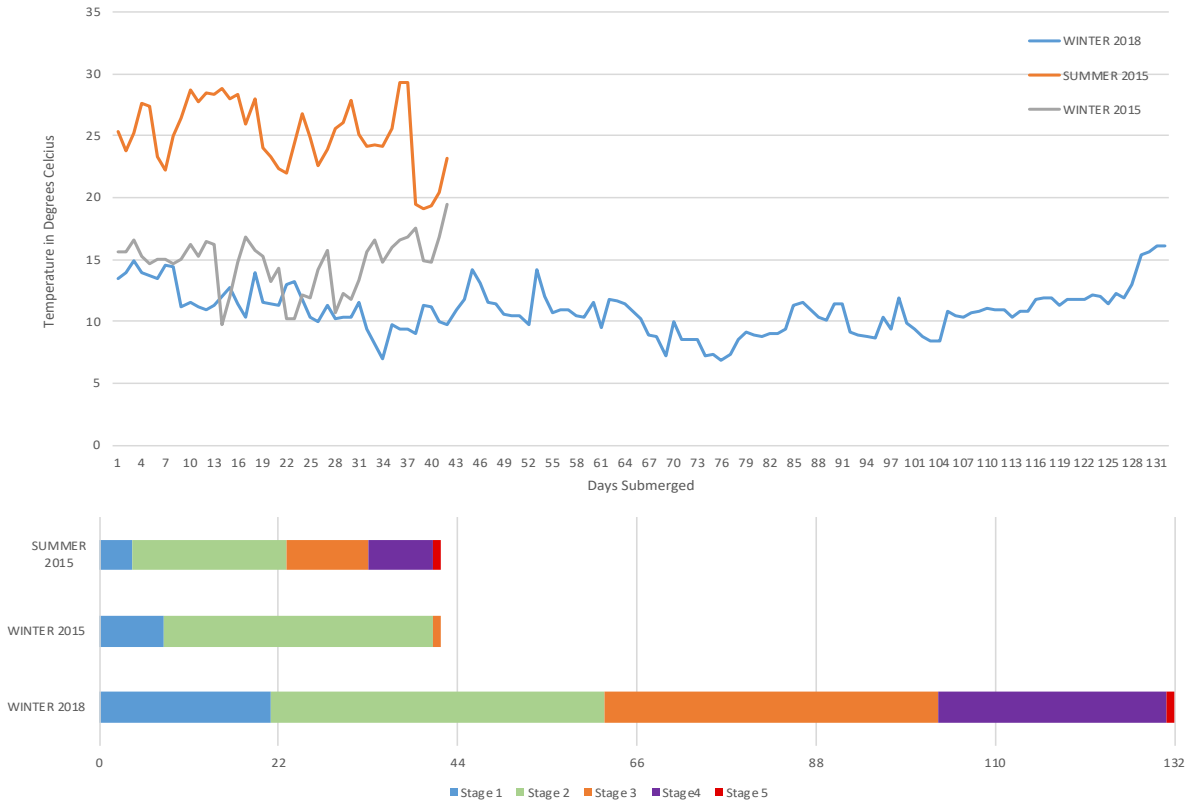


Figure 10: Daily temperature during the winter 2015 (blue), summer 2015 (orange) and winter 2018 (grey) studies and (below) the corresponding stage of decomposition of the piglets over each submersion period.

A comparison of all three studies was restricted to 42 days following submersion. The process of decomposition took the longest in winter 2018, followed by winter 2015 (incomplete decomposition) and then summer 2015 where decomposition was fastest. As the winter 2015 study was terminated on day 42 only the completed stages, 1 and 2, were comparable with the summer 2015 and winter 2018 studies. The start of the second and third stage of decomposition occurred after the shortest submersion period in the summer 2015 study, followed by the winter 2015 and took the longest in winter 2018.

ADD is a function of both temperature and time and therefore a lower temperature in the winter studies required a greater period of time for the same degree of decomposition to occur compared to the summer study.
The winter 2018 study had required the notably highest accumulated degree days (ADD) for the first stage of decomposition, (Figure 11). With regard to the two studies where decomposition was followed to completion, all stages of the winter 2018 study were completed over a longer period than those of the summer 2015 study.

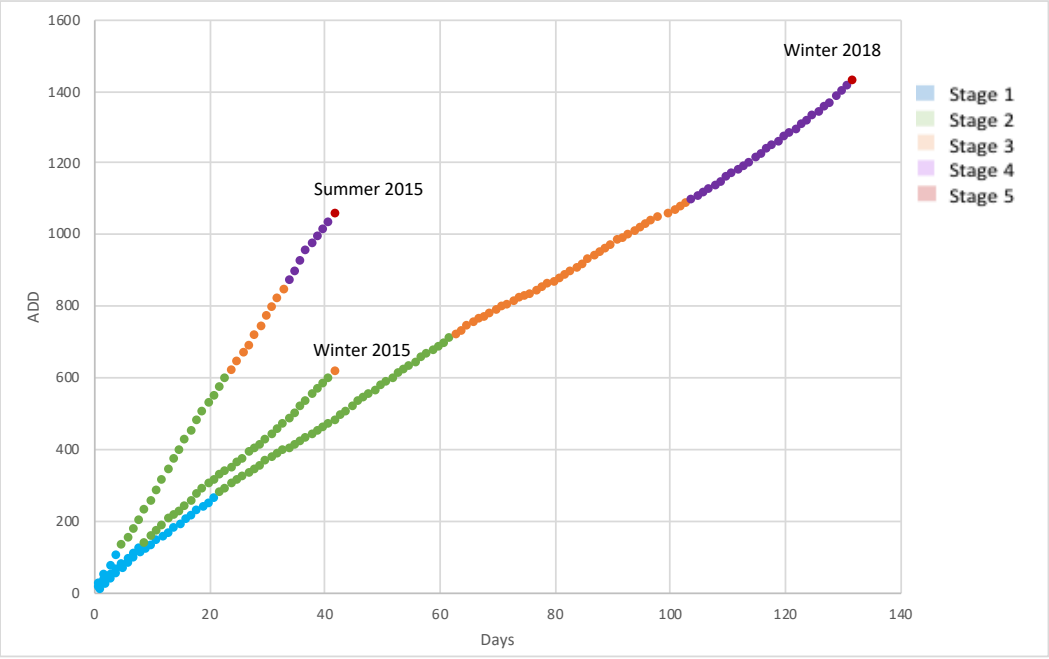


Figure 11: The progression of ADD over the submersion period of the three studies. The corresponding stage of decomposition of the studies are represented by the colour of the data point markers and as shown in the key on the right of the graph.

5.4 Biofilm

A biofilm layer, comprising unidentified bacteria and non-algal organisms, appeared on both the glass microscope slides and piglet carcass within two days of submersion. With increasing submersion period the biofilm layer became thicker and changed from a clear to a green mucus-like substance as algae colonised the substrate. The biofilm layer was more prevalent on the piglet carcasses than on the glass microscope slides and thicker on the summer substrates than those submerged in winter.

5.5 Algae and period of submersion

Algae were found to successfully colonise the carcasses throughout their decomposition (summer 2015 and winter 2018). It was only in the 2015 winter study that algal colonisation commenced in the second stage of decomposition. In the summer 2015 and winter 2018 studies algal colonisation commenced in the first stage of decomposition.

The most common algal taxa encountered in the three studies are illustrated in Appendix 6. Taxa that were not attached to the substrate but that settle on the substrate's surface were classified as planktonic and those that were attached to the substrate were classified as benthic.

5.5.1 Algal colonisation of the piglet substrates

5.5.1.1 Comparison of Algae Colonising the Piglet Substrate throughout decomposition (Summer 2015 and Winter 2018)

In the studies that followed the entire process of decomposition, algal diversity on the carcasses was greatest in winter (Figure 12 and Figure 13). In summer, *Chlorella* dominated throughout, whereas in winter, there was a more even spread of algal taxa. Benthic algae colonised the summer carcass after six days of submersion whereas the winter piglet required 12 days. Once colonised, there was a higher

diversity of algae on the winter piglet compared with that on the summer subject. In spite of the delayed colonisation by benthic algae in the winter study relative to the summer one, its diversity once colonised was highest.

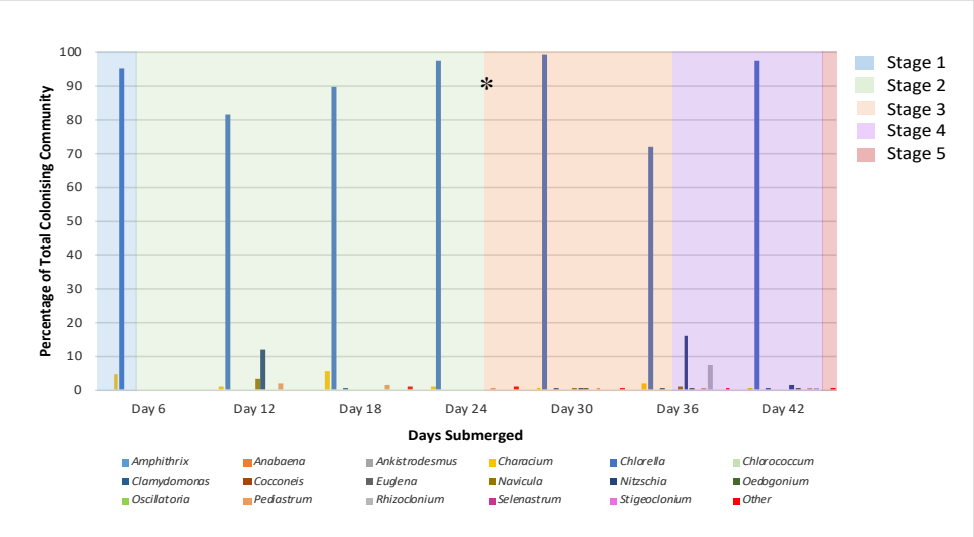


Figure 12:Dominant algal taxa colonising the piglet of the colonising community per sample date during the summer 2015 study. Taxa which account for less than 2% of the total colonising community were grouped under “Other”. The corresponding stage of decomposition of the piglet at any time in this series is represented by the background colour and as shown in the key on the right of the graph. The symbol * indicates the time when the abdomen ruptured.

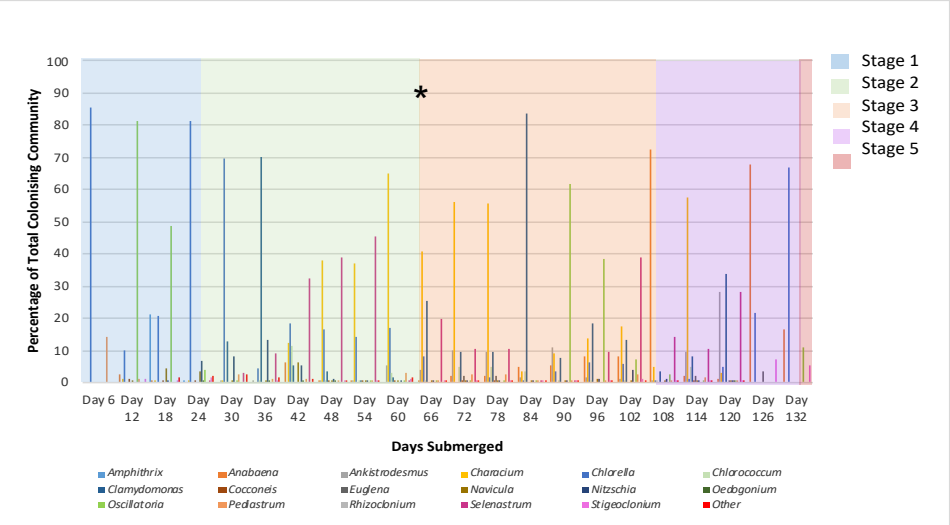


Figure 13: Dominant algal taxa colonising the piglet over time during the winter 2018 study. Taxa which account for less than 2% of the total colonising community were grouped under “Other”. The corresponding stage of decomposition of the piglet at any time in the piglet at any time in this series is represented by the background colour and as shown in the key on the right of the graph. The symbol * indicates the time when the abdomen ruptured.

There was greater diversity of benthic algae on the winter carcass from the time of colonisation than in the summer study (Figure 14 and Figure 15).
Diversity of benthic taxa in the summer study notably increased after the third stage of decomposition while planktonic diversity was consistent throughout submersion. The diversity of both benthic and planktonic alga decreased less following rupturing of the abdomen than it did following the increased sloughing of tissue that occurred in the latter parts of Stage 4.

The evaporation of water during the summer 2015 study required two supplementation events (days 18 and 32). The diversity of the benthic community dropped slightly on the sample date (day 24) following the first event, but was enhanced in the sample day (day 32) following the second event. The planktonic diversity was not affected by either event.

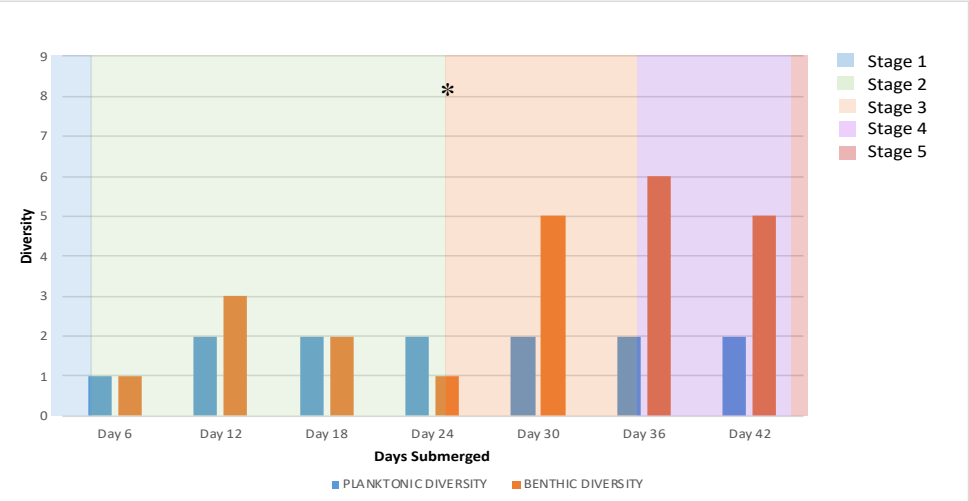


Figure 14: Diversity of benthic taxa colonising, and settled planktonic taxa on, the submerged piglet during the summer 2015 study. The corresponding stage of decomposition of the piglet at any time in this series is represented by the background colour and as shown in the key on the right of the graph. The symbol * indicates the time when the abdomen ruptured.

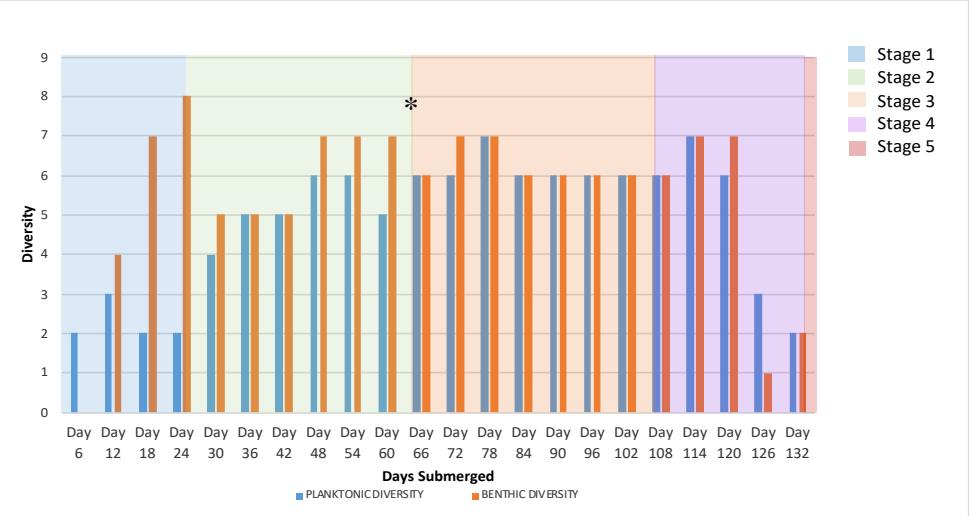


Figure 15: Diversity of benthic taxa colonising, and settled planktonic taxa on, the submerged piglet during the winter 2018 study. The corresponding stage of decomposition of the piglet at any time in this series is represented by the background colour and as shown in the key on the right of the graph. The symbol * indicates the time when the abdomen ruptured.

5.5.1.2 Comparison of algae colonising the winter 2015 and winter 2018 piglet substrate

In spite of the colder water temperature in 2018, the piglet was colonised by algae six days earlier than the 2015 piglet (Figures 16 and 17).

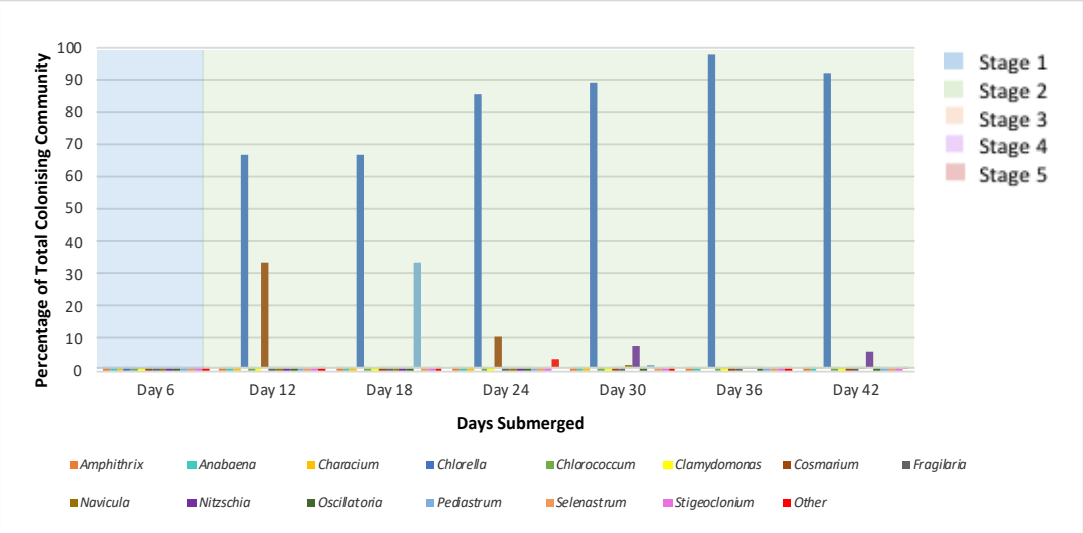


Figure 16: Dominant algal taxa colonising the piglet of the colonising community per sample date during the winter 2015 study. Taxa which account for less than 2% of the total colonising community were grouped under “Other”. The corresponding stage of decomposition of the piglet at any time in this series is represented by the background colour and as shown in the key on the right of the graph.

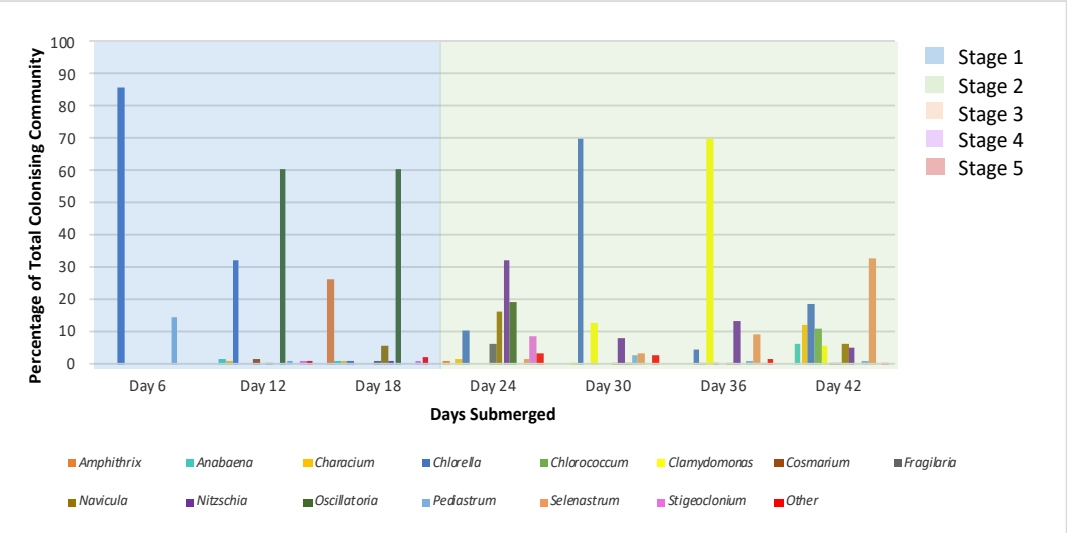


Figure 17: Dominant algal taxa colonising the piglet of the colonising community per sample date during the winter 2018 study. Taxa which account for less than 2% of the total colonising community were grouped under “Other”. The corresponding stage of decomposition of the piglet at any time in this series is represented by the background colour and as shown in the key on the right of the graph. The symbol * indicates the time when the abdomen ruptured.

No trend in the abundance of individual taxa with increasing PMSI could be discerned in either season, but there was an increase in general planktonic diversity. Apart from *Cosmarium*, the establishment of benthic algae in winter 2015 was relatively slower than that in the winter 2018 study, but once established, the number of benthic taxa remained fairly consistent in both studies (Figures 18 and 19).

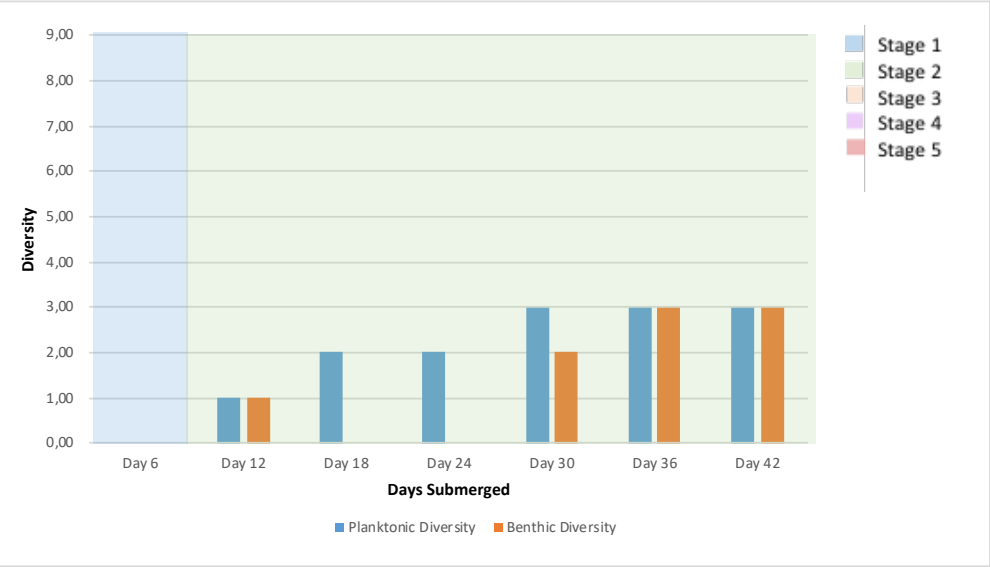


Figure 18: Diversity of benthic taxa colonising, and settled planktonic taxa on, the submerged piglet during the winter 2015 study. The corresponding stage of decomposition of the piglet at any time in this series is represented by the background colour and as shown in the key on the right of the graph. The symbol * indicates the time when the abdomen ruptured.

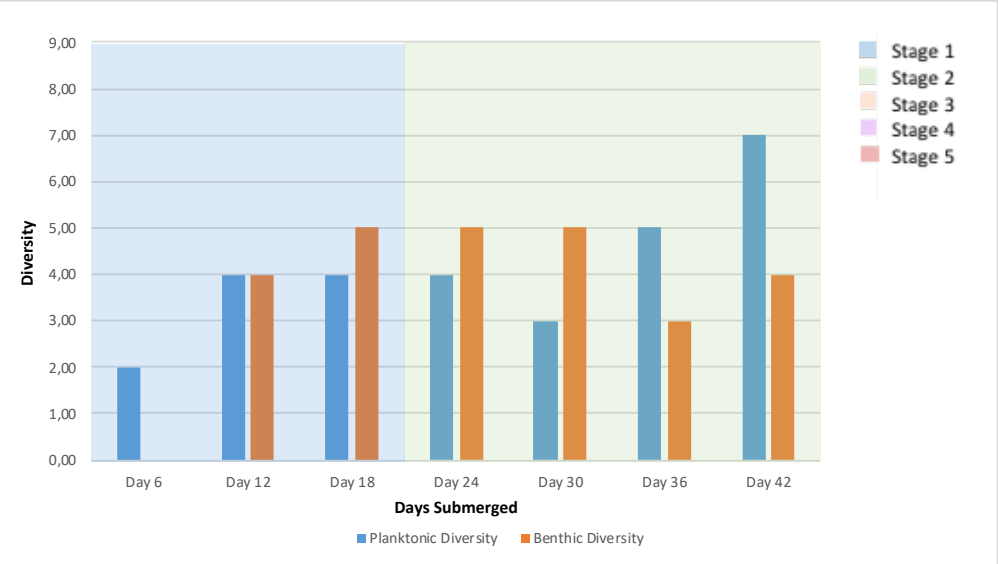


Figure 19: Diversity of benthic taxa colonising, and settled planktonic taxa on, the submerged piglet during the winter 2018 study. The corresponding stage of decomposition of the piglet at any time in this series is represented by the background colour and as shown in the key on the right of the graph. The symbol * indicates the time when the abdomen ruptured.

5.5.2 Shannon-Weiner Diversity Index of the Piglet

The Shannon Weiner Diversity Index (H), which considers both abundance and the even distribution (frequency) of colonising taxa, fluctuated in both the summer 2015 and winter 2018 studies where decomposition was completely followed. The slower decomposition in the winter 2018 study required markedly more time than the summer 2015 study. As a result, more samples were taken over the winter 2018 study period accounting for the greater number of data points and fluctuations in H than in the summer 2015 study. The most notable decreases in Shannon Weiner Diversity Index seen in both studies occurred in the third stage of decomposition and the second most notable decrease occurring at the start of the fifth stage of decomposition (Figure 20). The conceivable trend for winter 2018 illustrated in the graph below mimics the bimodal trend of H of the summer study.

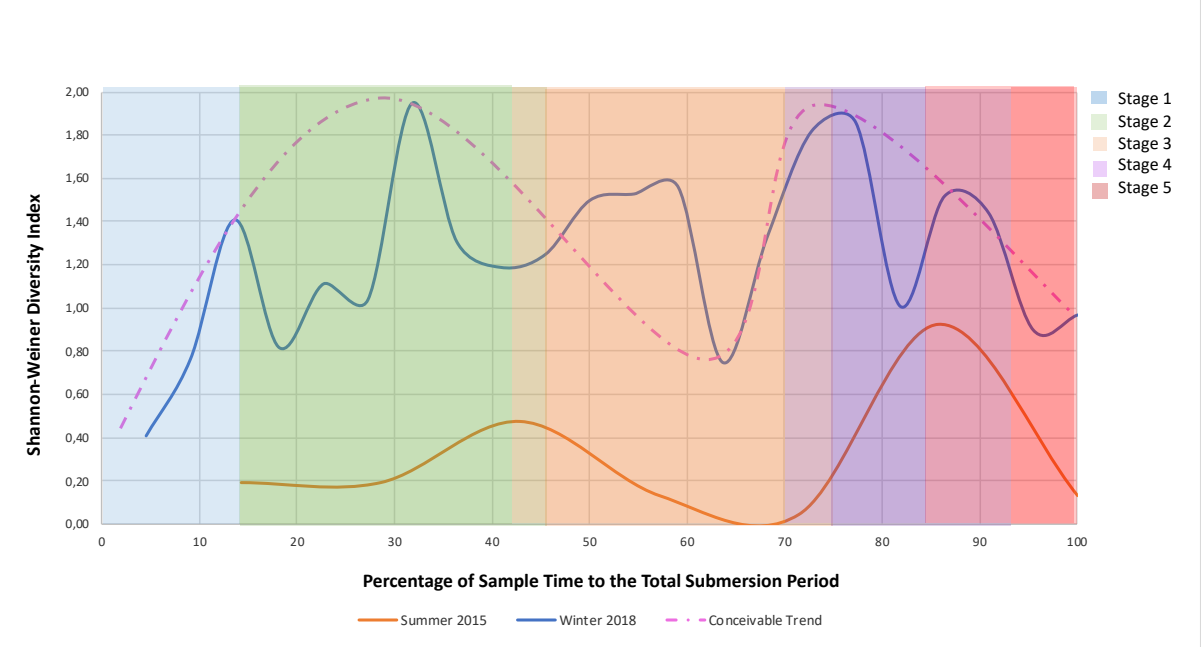


Figure 20: Shannon-Weiner Diversity Index (H) measuring the even distribution of colonising taxa every six days following submersion of piglets in summer 2015 and winter 2018 until their complete decomposition. The corresponding stage of decomposition of the piglet at each sample is represented by the background colour of the chart as indicated in the key on the right.

5.5.3 Relationship Between ADD, Shannon-Weiner Diversity Index and Stage of Decomposition

Regressional analyses between each of ADD, Shannon-Weiner Diversity Index or stage of decomposition and time showed that only ADD was significantly correlated with time in all three studies (Table 4). The stage of decomposition of only the winter 2015 study was credibly correlated with time and the remainder showed no reasonable correlation with time (Table 4).

Table 4: P Values of linear regression of dependant variables, ADD, Shannon-Weiner Index and stage of decomposition, to the independent variable, time. P values <0,05 (shaded) are statistically significant.

	P Value Summer 2015	P Value Winter 2015	P Value Winter 2018
ADD	1,556E-05	3,690E-05	3,688E-17
Shannon-Weiner Index	0,992	0,165	0,441
Stages of Decomposition	0,526	0,045	0,302

5.5.4 Relationship Between TAD score and Algal Diversity

No trends were seen between the response of benthic and planktonic algal diversity with a progressive increase in TADS (determined from the descriptive stages proposed by Heaton *et al.,* (2010)) in all three studies. This indicates TAD score cannot be used to determine the period of submersion from the fluctuating changes in algal diversity (Figure 21).

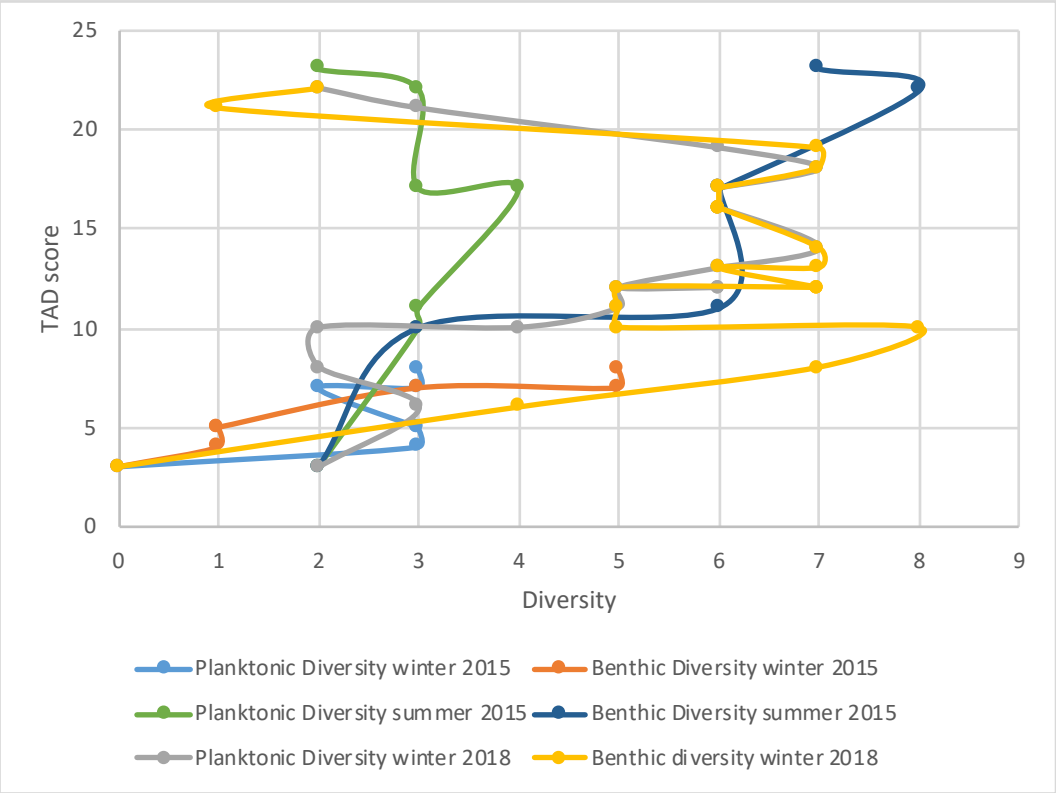


Figure 21: The change in planktonic and benthic algae colonising the piglet carcasses in summer and winter 2015 and winter 2018 over the progressive increasing total aquatic decomposition (TAD) score (derived from Heaton *et al.,* (2010))

5.5.5 Change in Algal Diversity and Abiotic Factors in Winter 2018

As algal diversity increased over the first two stages of decomposition the dissolved oxygen had a general trend of decreasing, (Figure 22). After the abdomen ruptured both the dissolved oxygen contents and pH notably decreased and thereafter levelled out. The most notable decrease in the Shannon-Weiner Index occurred during the third stage of decomposition after the aforementioned decrease in the pH level and dissolved oxygen.

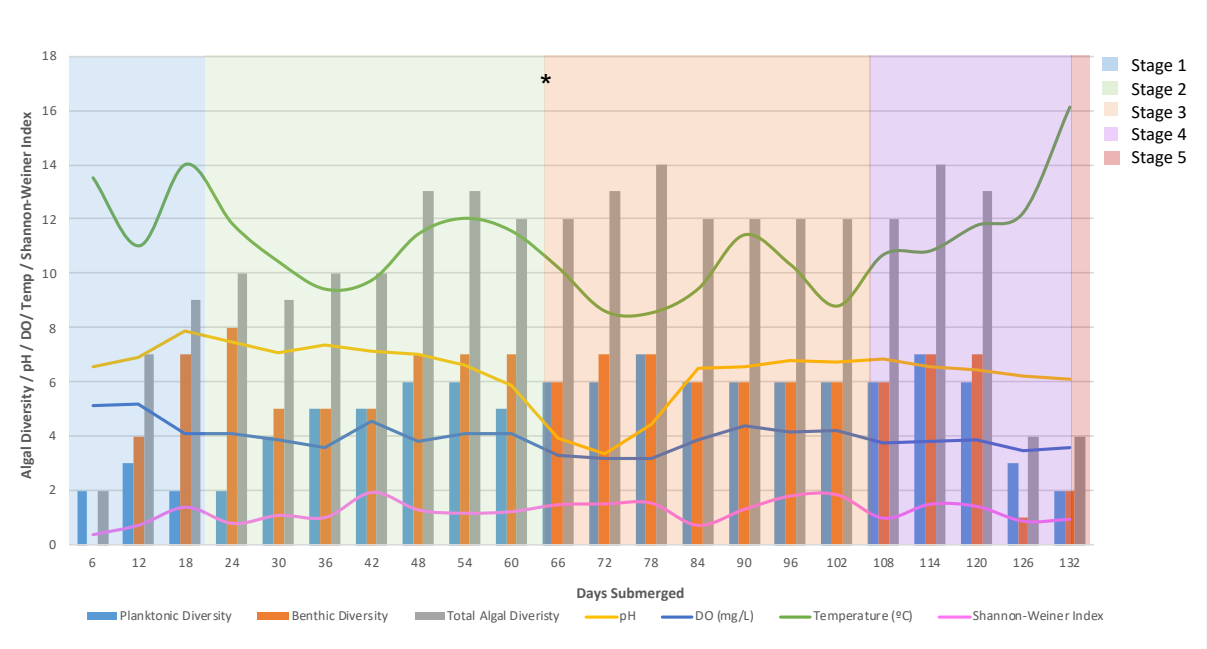


Figure 22: Change in algal diversity, Shannon-Weiner Index and abiotic factors (dissolved oxygen, temperature and pH) over every six days following submersion of the winter 2018 piglet. The corresponding stage of decomposition of the piglet at each sample is represented by the background colour of the chart as indicated in the key on the right. The symbol * indicates the time when the abdomen ruptured.

5.5.6 Algal Colonisation of the Glass Microscope Slides

5.5.6.1 Comparison of Algae Colonising the Winter 2015 and Winter 2018 Microscope Slides

A comparison of the colonisation of slides in the various seasons over the PMSI provides information on the background trends in change in the flora of the pond over the same time, without running the interference of the decomposition process (e.g.

potential anoxic conditions, sloughing of substrate, etc.). There was a notable difference in the taxa that colonised the glass microscope slides in these two studies. In spite of colder water temperatures in the winter of 2018, the microscope slides were colonised faster than those set out in the winter of 2015 (Figures 23 and 24).

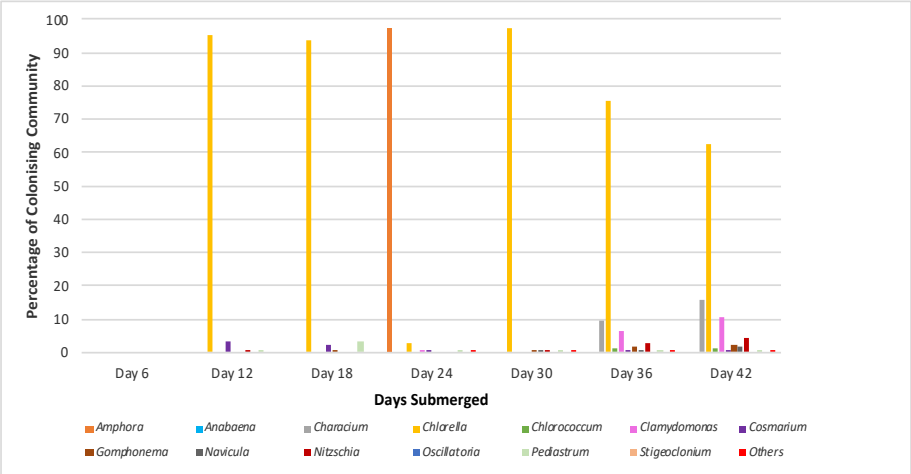


Figure 23: Dominant algal taxa colonising the glass microscope slides per sample date during the winter 2015 study. Taxa which account for less than 2% of the total colonising community were grouped under “Other”.

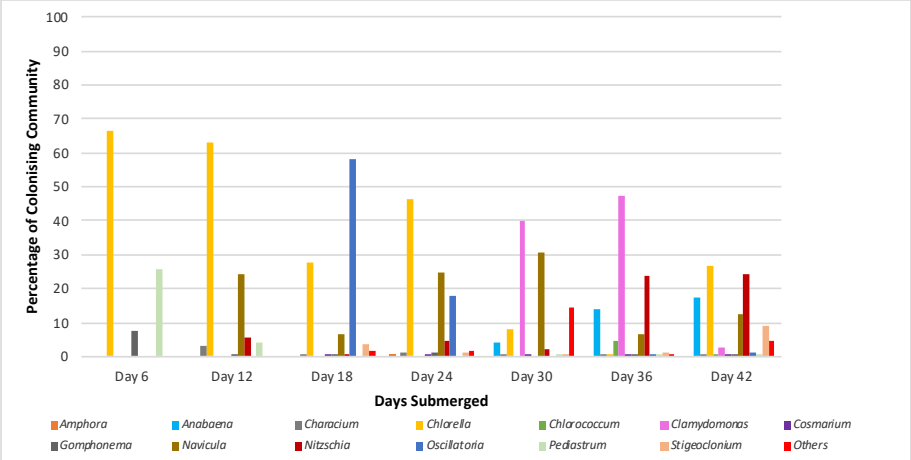


Figure 24: Dominant algal taxa colonising the glass microscope slides per sample date during the winter 2018 study. Taxa which account for less than 2% of the total colonising community were grouped under “Other”.

There was a general trend of increasing diversity of benthic algae over the submersion period for both winter studies (Figures 25 and 26).

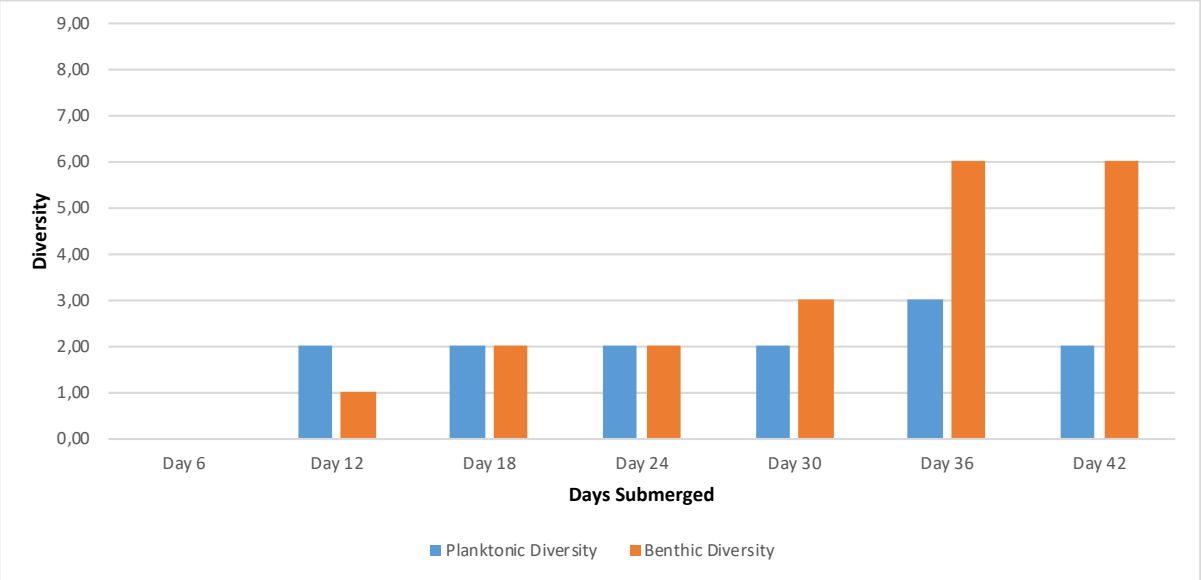


Figure 25: Diversity of benthic taxa colonising, and settled planktonic taxa on, the submerged glass microscope slides during the winter 2015 study.

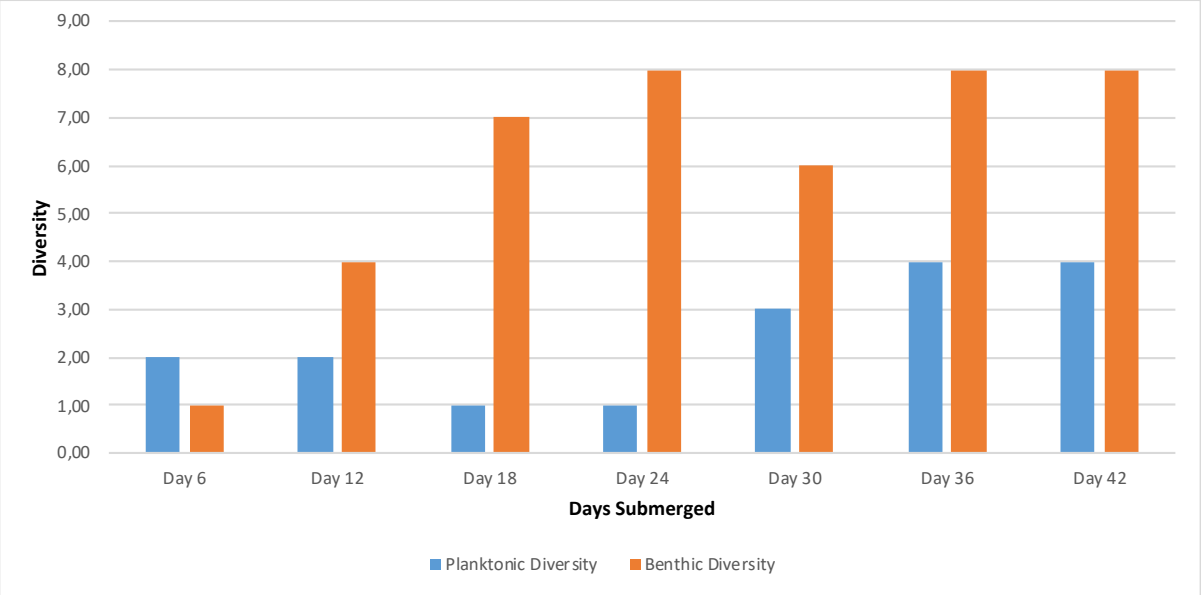


Figure 26: Diversity of benthic taxa colonising, and settled planktonic taxa on, the submerged glass microscope slides during the winter 2018 study.

5.5.6.2 Comparison of algae colonising the Microscope slides submerged in summer 2015 and winter 2015

There was a difference in the algae colonising the submerged slides between the seasons. Although *Chlorella* was the dominant species in both seasons, there was no trend in the relative abundance of each colonising taxon or the colonising algae of each sample over the submersion period (Figures 27 and 28). On day 24 in the winter study, there was a notable peak in *Cladophora* that subsequently disappeared.

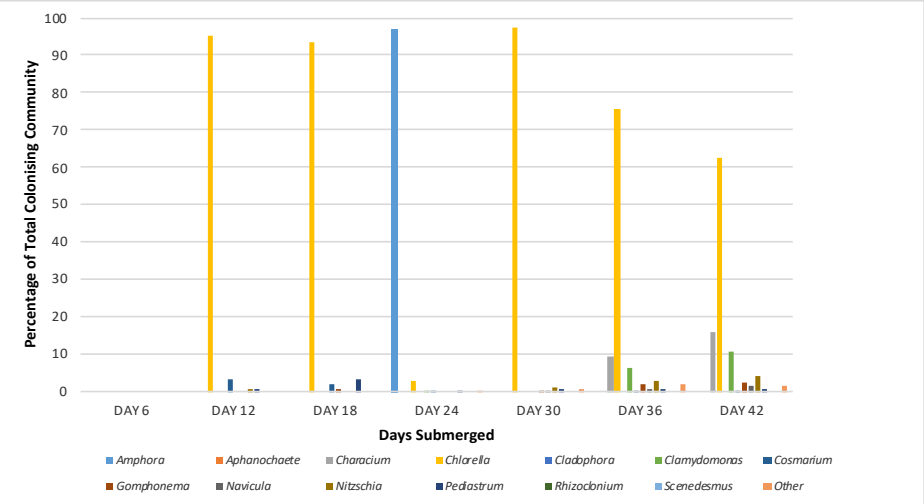


Figure 27: Dominant algal taxa colonising the glass microscope slides per sample date during the winter 2015 study. Taxa which account for less than 2% of the total colonising community were grouped under “Other”.

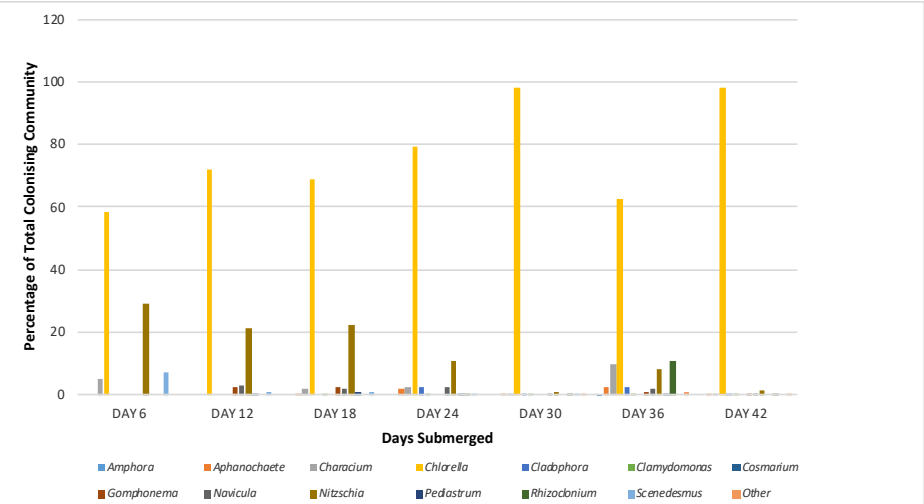


Figure 28: Dominant algal taxa colonising the glass microscope slides per sample date during the summer 2015 study. Taxa which account for less than 2% of the total colonising community were grouped under “Other”.

The summer microscope slides were colonised by benthic algae after a shorter submersion period compared with the microscope slides submersed in winter. The diversity of both the planktonic and benthic taxa was greater in summer 2015 than winter 2015. In both seasons there was a progressive increase in the diversity of benthic species colonising the microscope slides whereas the occurrence of planktonic taxon remained fairly constant throughout the submersion period (Figures 29 and 30).

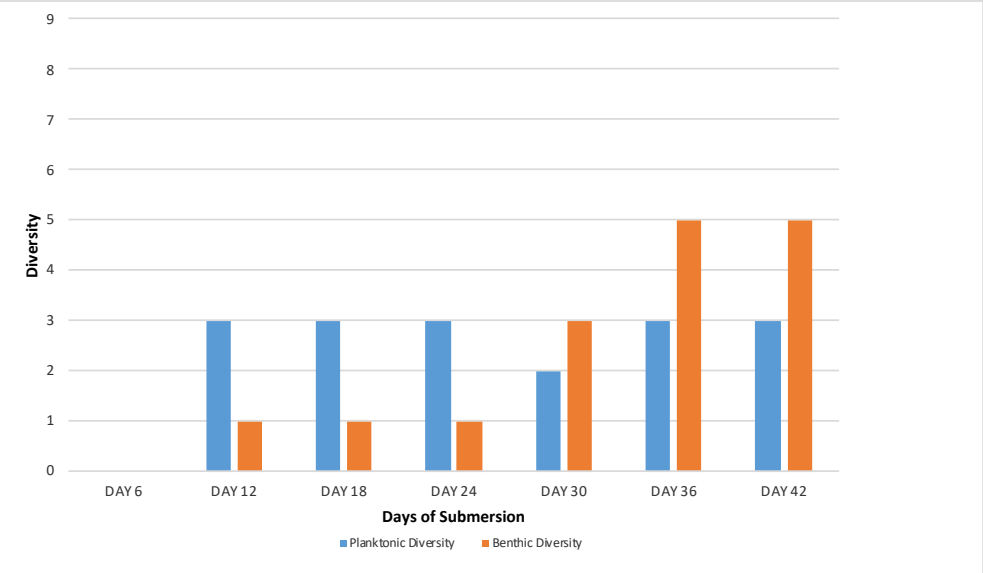


Figure 29: Diversity of benthic taxa colonising, and settled planktonic taxa on, the submerged glass microscope slides in the winter 2015 study.

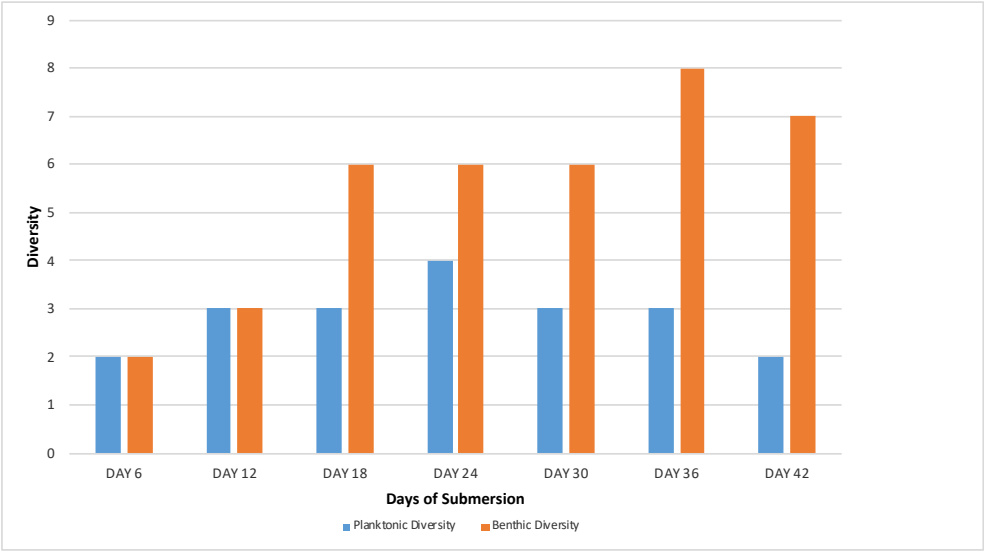


Figure 30: Diversity of benthic taxa colonising, and settled planktonic taxa on, the submerged glass microscope slides during the summer 2015 study.

5.5.7 Shannon-Weiner Diversity Index of the Glass Microscope Slides

The Shannon Weiner Diversity Index (H) fluctuated for both the summer 2015 and winter 2018 studies. In the winter 2018 study more samples were taken over the study period accounting for the greater number of data points and fluctuations in H than the summer 2015 study. The most notable decreases in diversity and dominance seen in both studies occurred after 70% of the study period (Figure 31).

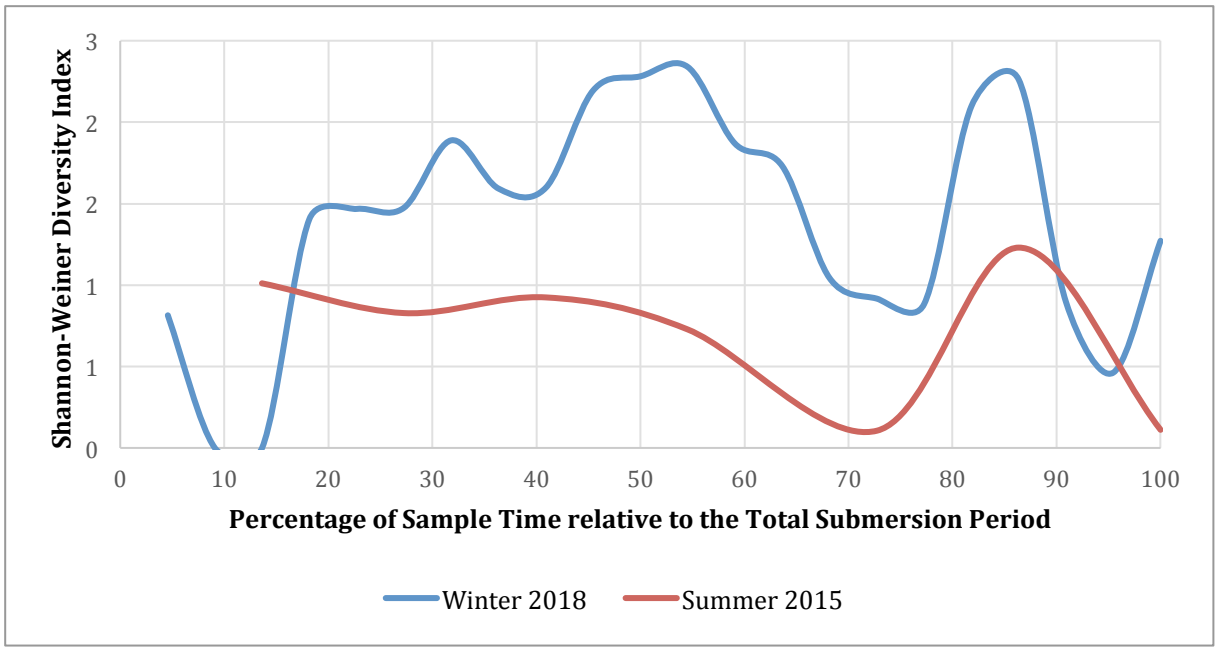


Figure 31: Shannon-Weiner Diversity Index (H) measuring the even distribution of colonising taxa every six days following submersion of the glass microscope slides in summer 2015 and winter 2018 over the total submersion period.

5.5.8 Sorenson's Similarity Index

The similarity in taxon composition (communities) on both substrates between summer and winter 2015 was determined through the Sorenson's Similarity Index (C_S). C_s increased as algal diversity increased on the winter 2015 substrates. Although the Sorenson's Similarity Index increased over the submersion period the community composition was never exactly the same on each sample date in winter 2015 and winter 2018. There was a greater similarity of the colonising community composition on each sample date for different seasons than for the same season in different years (Figure 32).

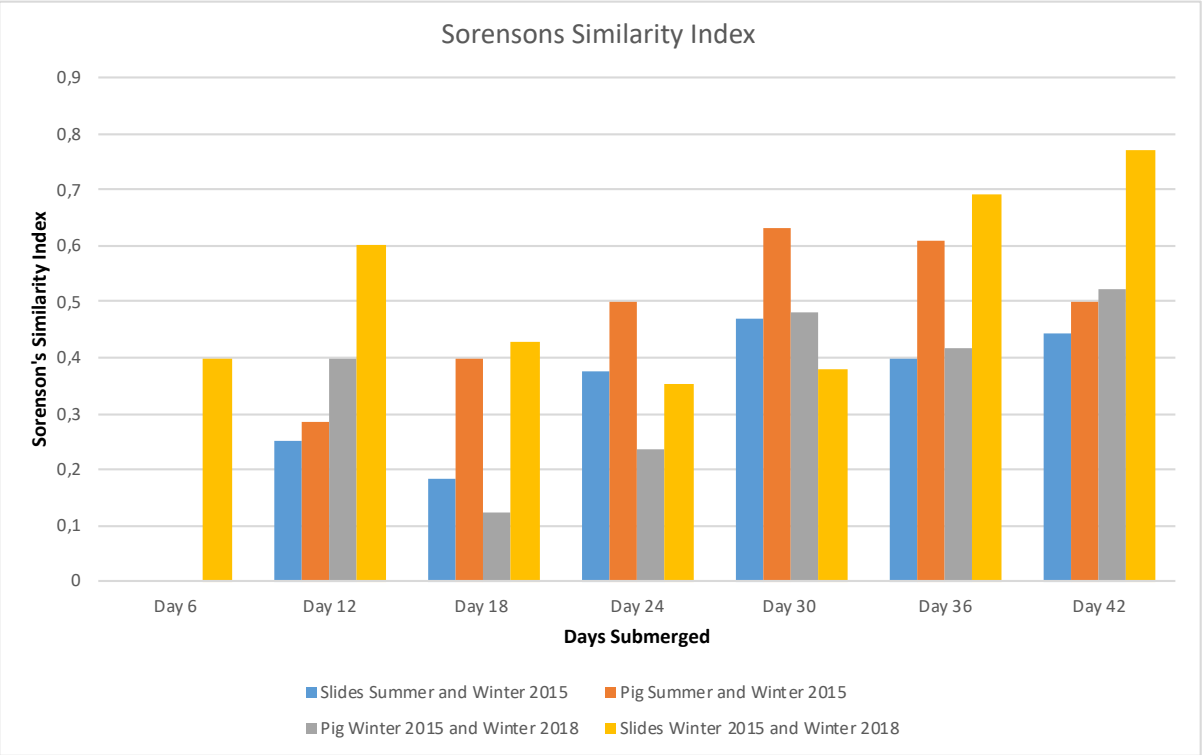


Figure 32: Sorenson's Similarity Index (Cs) comparing the algal community composition between years (winter 2015 and winter 2015) and seasons (winter 2015 and summer 2015) of algae colonising the piglet substrates and glass microscope slides every six days of submersion over a 42 day submersion period.

6 Discussion

6.1 Aquatic Decomposition

6.1.1 Identifiable Stages and Changes in Decomposition

Each stage of decomposition was discernible from the next and, although their names and characteristics varied slightly from study to study (Dickson *et al*., 2011; Haefner *et al.,* 2004; Zimmerman and Wallace, 2008), for the most part they resonated across studies. Subjective characteristics, such as the change in odour (following exposure of the carcass to the air; Dickson *et al*., 2011), would offer no assistance in defining the stage of decomposition of a recovered body as it would be subjective and it would not be possible for a forensic scientist to establish how these changed over the submersion period.

The changes in colour of the abdomen, firstly to green and then to purple-grey, were found to be identifiable characteristics for stage one and stage two respectively for all sample sets. The omission of or failure to note these changes in all previous research on decomposition of fully submerged carcasses (Anderson and Bell, 2014; Casamatta and Verb, 2000; Dickson *et al*., 2011; Haefner et al., 2004; Hobischak and Anderson, 2002) is surprising as both forms of discolouration were noted in studies on decomposing, floating carcasses (Ayers, 2010; Hobischak and Anderson, 1999). Different methods of monitoring change (Anderson and Bell, 2014; Dickson et al., 2010), as outlined below, may have obscured the relevance of such obvious markers in previous submerged studies. This may have been further confounded by the variation of analogue used. Carcasses with fur , such as the rats used by Casamatta and Verb (2000), would make changes to skin colour difficult to see and the use of only heads of adult pigs (Dickson *et al*., 2011) precluded any possibility of observing abdominal discolouration. Anderson and Bell (2014) were restricted to using monochrome cameras when monitoring decompositional changes and this prevented them from distinguishing any abdominal colour changes in their research. Abdominal discolouration is a definite and objective characteristic to identify the first

two stages of decomposition and it is recommended that allowance for colour detection be made in future work .

The rupturing of the abdominal wall, characteristic of stage three, was surprisingly not emphasized in previous studies on the decomposition of submerged carcasses (Anderson and Bell, 2014; Casamatta and Verb, 2000; Dickson *et al.,* 2011) but was identified as a defining characteristic of this stage in studies on floating bodies (Alley, 2007; Ayers, 2010; Hobischak and Anderson, 1999; Seet, 2010). The rupturing of the abdomen might have been overlooked in earlier research as it may have been difficult to detect whilst the carcass remained submerged. Instead of abdominal rupture, previous studies on the decomposition of submerged bodies used the occurrence of minor tissue loss of the lower legs and the thinning appearance of the carcass to signify the start of the third stage (Haefner *et al.,* 2004; Hobischak and Anderson, 2002; Zimmerman and Wallace, 2008). The use of limb tissue loss as an identifiable characteristic should be used with caution. As found in earlier research (De Donno *et al.,* 2014; Dickson *et al.,* 2011; Haglund, 1993; Heaton *et al.,* 2010; Petrik *et al.,* 2004) exposure of any part of the carcass to currents, scavengers and/or other environmental factors other than decomposition alone, can cause similar tissue loss. The rupturing of the abdomen along with tissue loss on the limbs in this study were definite indicators of the third stage. The abdomen proves to be a part of the body less susceptible, compared with face and distal limb tissue, to forces other than decomposition, be they biological or physical (Haglund, 1993; Haglund and Sorg, 2002; Renke, 2010). This supports the finding that the abdomen is a sensible place to follow changes in early decomposition up to, and including, the identification of the third stage. It is possible however that the rate of tissue loss and that abdominal rupturing was accelerated by the repeated removal of the carcasses from the water during sampling. Additional studies would be required to determine to what extent abdominal rupture remains independent of external forces, such as currents and scavenging activity, and what implications, if any, continuous handling has on decomposition.

Identifiable characteristics of the fourth and fifth stages are in keeping with earlier research (Hobischak and Anderson, 2002; Renke, 2010) indicating that these stages easily can be identified through objective characteristics.

Interestingly, the decompositional changes and the number of stages for submerged decomposition were similar to those in studies on decomposition of bodies in floating aquatic and terrestrial environs (Chin *et al.,* 2008; Hau *et al.,* 2014). The differences in the time taken for each stage to complete highlights the impact of the environment on the process of decomposition.

With the limited common data available from the two winter studies, it was clear that it took longer for stage one and two to be complete in the winter 2018 study than in the winter 2015 study. This disparity could be due to a higher load of bacteria inside the 2015 piglet carcass, resulting in a more rapid rate of decomposition. However it is more likely that this is the result of differences in the temperatures experienced in the two winters. Temperatures below 10°C have been found to significantly retard decomposer activity (Higgs and Pokines, 2002; Zhou and Byard, 2010). Temperatures in 2018 dipped as low as 7°C (Stage 2- Bloated Stage) and on average were lower during stage one and two in the winter 2015 study compared with the winter 2015 study, so such a disparity is unsurprising. The relationship between temperature and decomposition is not linear, so, when estimating PMSI from ADD, one would need to consider whether the body was exposed to temperatures lower than the threshold at which decomposer activity ceases. This nonlinear relationship together with the effect of other environmental conditions on the rate of decomposition will impact on the duration or ADD of each stage of decomposition across seasons even though decomposition occurred in a similar manner. Such possible variation limits direct comparisons of the time and ADD for each stage of decomposition between seasons, year and different aquatic locations. If the effect that environmental temperature had on the degree of decomposition of a body could be accounted for through the use of ADD, one can then begin to consider what and how other environmental factors affected decomposition of the body. It is important to consider the broader environmental factors to which a body is exposed when classifying the stage of decomposition. For example, currents, disturbance or presence of scavengers, could accelerate the rate of decomposition of the exposed part of the body (Haefner *et al.,* 2004; Haglund and Sorg, 2002). This has the

potential to result in a body with a mosaic of decompositional characteristics, which would make its classification to a specific stage of decomposition challenging. Renke (2010) also highlighted the importance of considering environmental factors which alter decomposition when determining the stage in aquatic environments. Additional research considering the effect of specific environmental conditions in isolation and collectively will determine whether their effect/s on decomposition have any pattern. The application of such findings in the field for the forensic scientist, however, remain quite limited. Unless the environmental factors a body was exposed to remained constant during submersion and once the body was recovered, it would not be possible for a forensic scientist to determine by which particular environmental factor(s) and to what degree the body had been altered.

Of the limited number of aquatic decomposition studies, few focus on the decomposition of submerged bodies. Differing approaches to such studies and the analogues used make it difficult to directly relate the stages of decomposition from one study to the next. The association of abdominal colouring to the first two stages and the rupturing of the abdominal wall to the third stage of decomposition offer more objective standards to identify a stage. As the decompositional changes and the number of stages found here were similar to that of terrestrial decomposition studies (Chin *et al.,* 2008; Hau *et al.,* 2014) there is no need to alter the method of identifying the stage of decomposition for an aquatic environment. Identifying the stage of decomposition of a recovered body is critical to the determination of the PMSI.

6.1.2 Accumulative Degree Days (ADD) Between Seasons and Previous Aquatic Decomposition Studies

6.1.2.1 ADD Between Samples

Decomposition took longer in colder water compared with that in warmer water. This is not surprising and is in keeping with general findings of earlier research (Allaire, 2002; Evans, 2010; Petrik *et al.,* 2004; Renke 2010) that higher temperatures have a positive effect on the rate of decomposition. This signifies that environmental temperature is indeed a major environmental factor affecting the decomposition of a submerged body. Any difference in water temperature between seasons, or between years for the same season, impacted on the length of the submersion period for each

decompositional stage, but the ADD values were similar. Any differences in both the ADD and the timing of each stage of decomposition are to be expected as there are other environmental factors, other than temperature, to which the carcass is exposed . The PMSI is therefore a range and not an absolute value and is due to many different environmental factors affecting decomposition. It is also dependent on the frequency of observations: An increase in frequency offers a more precise timing of transition between stages of decomposition. Because the duration of any stage is used to calculate the ADD, the ADD will be a range. The estimation of PMSI of a body can be narrowed to the overlapping ranges of the PMSI derived from the ADD and that derived from any other biological indicator, such as algae.

The different ADD of each stage of decomposition in each sample sets indicates either that the relationship between ADD and decomposition is not directly proportional or that additional factors (abiotic and biotic) have a synergistic effect on decomposition. Such a disproportionate relationship between ADD and rate of decomposition has been found before (Haglund and Sorg, 2001; Myskowiak *et al*., 2010; Zhou and Byard, 2010). In these studies water temperatures below 10° Celsius retarded decomposer activity. The colder water temperatures in the winter 2018 study can account for the higher ADD through decreased decomposer activity. The range of PMSI determined from ADD is narrower where temperature favours decomposer activity.

The absence of research concerning decomposer activity in aquatic environments highlights the need for the inclusion of this aspect in future research. Research would be needed to establish the minimum and maximum temperature threshold for decomposer activity in various aquatic environments. Understanding the effect of temperature on decomposer activity will allow this aspect to be factored in and provide a barrower PMSI range. As an aside, a difference in the ADD between samples can also be because the effect of abiotic and biotic factors, e.g. currents and scavenger activity, as proposed by Heaton *et al*. (2010) and De Donno *et al.* (2014). A difference in environmental factors between the two winter studies would affect the biota, including decomposers, and influence the rate of decomposition. Understanding how environmental factors, individually and collectively, affect decomposition, however, would be of limited help to a forensic scientist estimating the PMSI as s/he would not be able to determine the history of the various

environmental factors to which the recovered body was exposed, especially those which vary markedly. Because of this, s/he would be unable to correct the estimate of PMSI appropriately. Additional research should rather set out to narrow the range of ADD by increasing the sample size in different seasons and environs.

6.1.2.2 ADD Across Aquatic Studies

A comparison of ADD from this and other submerged decomposition in similar pond studies where current had little impact (Figure 33), reinforces the earlier observation that the ADD for each stage of decomposition is a range rather than an absolute value. The broad range for the ADD of this study and earlier studies with similar environmental conditions supports the findings of Heaton *et al*. (2010) and De Donno *et al.* (2014) that the ADD alone is an unpredictable determinant for the range of PMSI. It is noted that the environment and methods used in this study are different to those of earlier research (Zimmerman and Wallace (2008), that used piglet carcasses in brackish ponds in Delaware, Haefner (2004) used pig carcasses in a stream in Pennsylvania and in the current study piglet carcasses were used and submerged in a pond in South Africa. The difference in analogues and environment between these two studies most likely contributed to the difference in the ADD seen. Such a comparison however should not be avoided as it is unlikely that an environment into which a body was submerged would be directly comparable to the environment used in earlier research and the data from which an approximate PMSI is being extrapolated. As mentioned above, untraceable environmental factors acting on a decomposing body will be of no use in estimating the PMSI. It is therefore recommended that research focus on traceable factors, most commonly temperature, and narrow the range of ADD per stage by increasing the sample size but still consider the ADD as a range and not an exact value. The same issues which are problematic for the forensic scientists in determining what environmental factors a body was exposed to are applicable here. If more studies are done controlling the various environmental factors to which a body is exposed it may be possible to see what effect each has on decomposition and factor these in estimates of the ADD and PMSI. Other factors associated with the remains, such as colonisers, may become important is determining the PMSI.

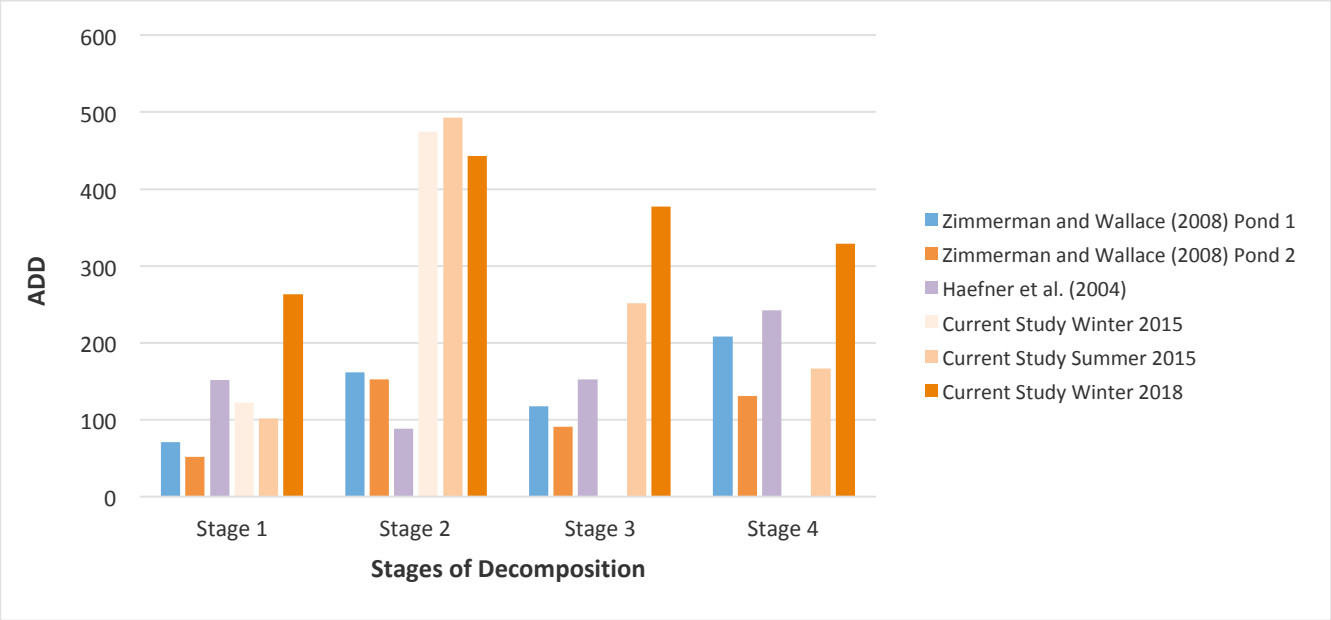


Figure 33: Comparison of ADD per stage of decomposition across studies conducted in fresh water ponds.

In conclusion, with regard to ADD, its range is a function of aquatic environmental factors that would be of limited use to a forensic scientist due to the broad range of derived PMSI. Even under similar environmental conditions the ADD determined for each stage of decomposition will differ. The potentially broad range of the ADD per stage in aquatic environments makes for a much more diffuse estimate of PMSI and prevents a forensic scientist from being able to use the ADD determined from one standing aquatic environment to estimate the PMSI of a body recovered in another. Future research would need to determine the ADD per stage for varying aquatic environments in order to account for the effect of environmental factors on decomposition and narrow the range of ADD. Considering ADD in relation in changes in succession of any epizoic organisms may offer a more complete picture of the submersion period and may prove a promising approach to estimating the PMSI.

6.1.3 TADS and ADD to Calculate the PMSI

Plotting the TADS (determined from the characteristics provided by Heaton *et al.,* (2010) against $\log_{10}$ADD illustrated that the slope of $\log_{10}$ADD versus TADS was the steepest in the summer 2015 study. The equations and slope produced in this study not only differed to each other but were also different to those produced in other studies (Heaton *et al.,* 2010; Humphreys *et al.,* 2013; Reijnen *et al.,* 2018). Different equations and slopes were expected between this study another other research as the methods differed. Human corpses were used by Heaton *et al*. (2010) and Reijnen *et al.* (2018) and piglet analogues were used in the current study and that of Humphreys *et al.* (2013). The studies were all conducted in different freshwater environments (UK rivers in Heaton *et al.,* 2010), and spanned a variety of freshwater locations, including ditches, canals, channels rivers, ponds, puddles and lakes, in Amsterdam (Reijnen *et al.,* 2018), a man made reservoir (Humphreys *et al.,* 2013) and a freshwater pond in Johannesburg, South Africa (current study). The different equations produced in the three studies of this study however indicated that, even for the same location, the relationship between ADD and decomposition (whether it be TADS or stages of decomposition) will differ because of season and as a result of the environmental conditions to which the decomposing body is exposed. For the equation derived from the TADS and ADD to be useful in determining the PMSI, it would need to be determined for each season and each location where a body is found.

What makes the approach of using TAD score appealing in the determination of PMSI is that both the degree of decomposition and the ADD are used . Furthermore, the water temperature in the general area where the body was found can be used. Therefore, the model can be used to estimate the PMSI even if the exact temperature of the water in which a deceased person is found is unknown (Reijnen *et al.,* 2018). When the linear relationship is determined for a particular location from the TAD score and the known ADD, the PMSI can be inferred from this equation by taking into account the average daily temperatures experienced from the date the body was recovered until the estimated ADD is reached.

Considering the actual ADD and that calculated from the derived equation, it was noted the calculated ADD differs to the actual ADD with increasing TAD score. This

finding corroborates that which was reported by de Donno *et al.,* (2014) that the greater the PMSI, the less accurate the reconstruction of the thermal history. These findings are in line with those of Fink (2017). It is recommended that alterations be conducted to the aquatic decompositional scoring method when attempting to score carcasses with low ADD (such as in colder water temperature) or high ADD (more advanced degree of decomposition). Although a promising approach, the TADS model suffers the same limitations as that involving ADD alone: decomposition is not just a function of temperature. The process of decomposition is influenced by an array of environmental factors and for a more accurate PMSI to be determined, more of these factors will need to be considered.

When the change in algal diversity was incorporated into the TAD score, no identifiable trends were seen in any of the three studies. Measurable changes in algae were found to repeat themselves over the submersion period. Because of this you need to be careful which of the recurring values of algal measures is used to determine the PMSI. A way to deal with this is to select the value which relates to that particular degree of decomposition. The absence of trends in the TAD score across studies prevents it being used to identify the relevant period of the changes in the algal community (represented in the bimodal change of Shannon Weiner Index, community composition or diversity) and therefore the PMSI. It can however be used to increase the accuracy of the PMSI determined when used in conjunction with another method, such as algal growth, as more factors are considered.

6.2 Potential Measures of Algal Colonisation Useful in PMSI Estimation

6.2.1 Individual Taxa as a PMSI Estimator

Individual taxa cannot be used as a marker for PMSI because, as indicated in the present study, no obvious pattern in their presence/absence is apparent throughout the process of decomposition, nor can they reasonably be expected to be present in different seasons, different years (current study) or all locations (Whitford, 1960; Casamatta and Verb 2000; Zimmerman and Wallace, 2008). Restricting analysis to the occurrence of specific organisms (Casamatta and Verb, 2000) or a group of algal taxa, as was attempted with diatoms (Zimmerman and Wallace, 2008), limits the

potential of algae as PMSI estimates. Focusing on a smaller component of the colonising community prevents detection of changes of the entire colonising community and the relevance of such to PMSI determination would be overlooked. The intermittent presence of specific taxa over this study makes it inappropriate to consider single taxa when attempting to ascertain PMSI as it relates to multiple periods of submersion.

Clearly, the intermittent occurrence and variability of individual taxa in different seasons, years and aquatic locations makes any consideration of such taxa ineffective in estimating the PMSI.

6.2.2 Algal Succession

Given the fact that individual taxa are of no use, we should turn our attention to broader changes in the colonising community. The continuous colonisation of the carcasses, in spite of the decomposition of these, reinforces the theory that algae have the potential to be used to determine PMSI (Casamatta and Verb, 2000; Keiper and Casamatta, 2001; Merritt and Wallace, 2000). The patterns in succession of colonising algae seen in this study may offer a measurable approach to estimate the PMSI of a recovered body.

A difference in the PMSI in the initial stages of the two winter studies up to the point at which the substrates were first colonised was unexpected. The reason for the absence of algae on day 6 of the winter 2015 study is unknown. It is possible, based on the fact that no algae were found in the first sample of each study, that the result was due to human error. Although no error was noted during collection, it is possible that the water was agitated prior to sampling and that any algae that had settled onto the substrates were dislodged.

The pioneering species in the present study were capable of rapidly colonising the substrate because they were able to rapidly divide, settle or attach to the substrate. These include forms that glide along the substrate surface (Stevenson *et al.,* 2011) and fast-growing, stalked forms (Casamatta and Verb, 2000; Stevenson *et al.,* 2011; Vermaat, 2005). Later colonisers, in this study typified by filamentous forms, require more time because their development is tardier and/or episodic, rather than continual (Casamatta and Verb, 2000; Townsend *et al*., 2008). In particular, the timing of zoospore or gamete production has an impact on their colonisation while unicellular forms, typical of pioneers, generally divide on a diel cycle, and rapidly develop large, dispersed populations (Townsend *et al.,* 2008). The additional time required for the establishment of slower-developing, more complex growth forms may be of use as a marker of PMSI. A similar delay noted in earlier research (Casamatta and Verb, 2000; Sorg *et al*., 1997; Zimmerman and Wallace, 2008) supports this. The occurrence of late colonising forms on a body can therefore indicate that a body has been submerged for a long enough period to be conditioned by these, while a body only colonised by unicellular pioneering forms indicates a shorter submersion period. While a forensic scientist may not be able to definitively put a specific time to the submersion interval, s/he would at least be able to determine whether a threshold has been attained beyond which more established forms appear and before which only transitory forms are encountered. The submersion period associated with the arrival of specific marker taxa will differ between season and year, as evidenced by the results of this study. Such threshold points would need to be ascertained for different seasons and for different aquatic environments.

In spite of the glass microscope slide's surface being so different to that of the piglet carcass, the change in the diversity of the colonising community followed the expected bell-shaped curve that would occur for a dominance controlled community (Townsend *et al.,* 2008). Although a difference in the substrate's surface must impact on the community composition as certain taxa are better suited for different substrate types (Hobischak and Anderson, 2002), little to no change in the diversity of the colonising community was noted. Spatially heterogenous substrates provide more attachment sites (Keiper and Casamatta, 2001) and provide protection from disturbances (Schneck and Melo, 2011) and therefore tend to have an overall more diverse colonising community. For this reason the algal communities on the glass

microscope slides and piglet carcasses were not directly compared in this study but rather the general trends in diversity were considered for each substrate type.

The change in the colonizing taxa over the submersion period supports the theory of succession in dominance-controlled communities (Townsend *et al.,* 2008) where diversity is initially low. This is because the majority of the substrate is open for colonisation. Pioneering species lead colonisation because of their ability to rapidly colonise and grow. This required set of characteristics is shared by a rather limited pool of organisms. With intermediate disturbance, e.g. tissue loss, newly uncolonized flesh as a result of the tissue loss can be colonised by more competitive species. These additional species, if they outcompete some early pioneers, at least maintain or add to the diversity. Later, if there is no disturbance, the diversity will start to decrease as stronger competitors establish and displace many of the earlier colonisers (Townsend *et al.,* 2008). Zimmerman and Wallace, (2008) and the current study support such a bell-shaped response in diversity with time. This trend is a potentially useful approach to estimate the PMSI of a recovered body where high algal diversity (>8; see Figure 5 below) is indicative of mid-succession changes and related to stages two and three in winter 2018 or stages three and four in summer 2015. However, for this to be useful to the forensic scientist, the absolute value of diversity is required and this study showed season has an impact on this absolute value of diversity at each stage of decomposition. Therefore, season must be considered when using algal diversity measures as the maximum diversity (maximum point of the bell-curve) will differ according to season. The difference in the absolute value of the maximum diversity per stage across seasons may be due to an overall lower diversity in summer. This indicated the impact of seasons on this approach. An alternative possibility is that the lack of replication resulted in skewed data set. Additional research would be needed to confirm such bell-shaped trends in the change of diversity over time and the impact of seasons on this.

Previous freshwater studies by Casamatta and Verb (2000) excluded the analysis of decomposition in their study. This prevented comparison to the current study, which is the only study to date to consider the change in the colonising community and decomposition of the carcass in a freshwater environment. Comparisons of the current study (freshwater) were limited to earlier research conducted in brackish water (Zimmerman and Wallace, 2008). Data provided by Zimmerman and Wallace

(2008) were included in a comparative analysis of currents trends in diversity (Figure 34). The trend in diversity in summer 2015 obviously differs from the remainder, presumably because the warmer temperatures favoured rapid decomposition before the algae get a chance to respond. If it is excluded, a trend of diversity per stage becomes evident: a diversity value ranging from 11 to 13 is indicative of stage 2 (Bloated Stage), from 9 to 12 is indicative of stage 3 (Floating Decay stage), from 8 and 10 is indicative of stage 4 (Advanced Floating Decay Stage) and from 4 and 7 is indicative of stage 5 (Sunken Remains Stage). The identification of a range of algal diversity per stage means that it be used to determine the stage of decomposition. However, future research would be needed to confirm whether such a comparison is true for all aquatic environments and in all seasons.

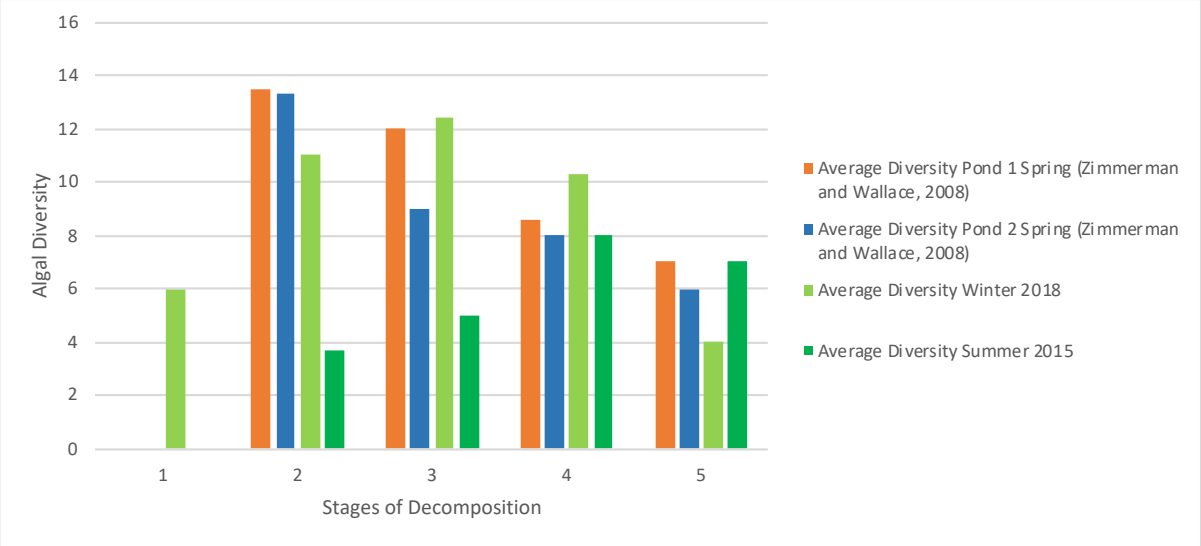


Figure 34: Comparative algal diversity per stage of decomposition in brackish water (Zimmerman and Wallace, 2008) and fresh, standing water (current study).

The PMSI (time) can be determined when algal diversity, stage of decomposition and ADD are considered (Figure 35). The stage of decomposition can be determined through visual assessment and corroborated by determining the algal diversity on the body at the time of its recovery. This stage is characterised by a range in ADD, so once this is determined, together with the temperature to which the body was exposed, (determined from local climatic data), a corresponding range in PMSI can be determined. The climatic data would need to be adjusted for water of pertinent volume and depth as it pertains to air. The range of the derived PMSI would be broader as it is a result of the compound of the range of ADD and of algal diversity

relative to each stage of decomposition. The relevant ranges can be reduced through increasing the sample sizes in future research when analysing these and factoring in, where possible, environmental factors that may have contributed to a broader range of PMSI.

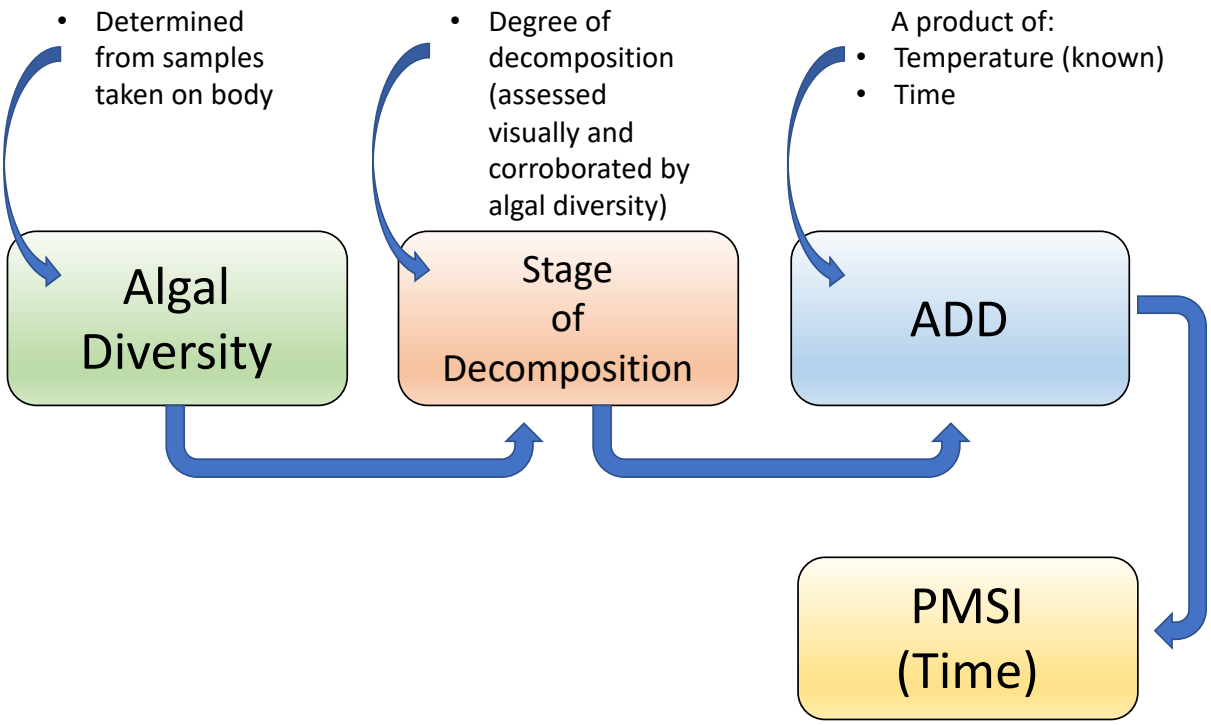


Figure 35: Illustration of the process of using algal diversity, stage of decomposition and ADD to determine the PMSI. Bold arrows indicate the data from one box may be used to inform those of the adjoining downstream box.

In conclusion, the colonisation and succession of algae on a carcass supports the notion that algae have the potential to be used to determine the PMSI. The comparable changes in diversity over time and the presence of growth forms are two approaches by which the PMSI may be determined through algae. However, additional research is required to confirm the usefulness of these two approaches, particularly as they pertain to different aquatic environments and to different seasons. Nevertheless, the current findings do demonstrate promise.

6.2.3 The Relationship of Algae, ADD and Decomposition to Time

Of the variables ‘stages of decomposition’, ‘Shannon-Weiner Index’ and ‘ADD’, ADD was the only one found to have statistical significance relative to the period of submersion in all of the studies. Being statistically significant means that the difference between the variables is unlikely to be attributed to chance and should be considered. In itself this is hardly surprising as it is a product of time and temperature. The lack of statistical significance with respect to the Shannon-Weiner Diversity Index in all of the studies, and over all five stages of decomposition in the summer and winter 2015 studies, does not occlude a relationship of these variables with PMSI. It is possible that each of their relationships are not straightforward. There are a myriad of factors, both abiotic and biotic, that can influence the relationship between these and time. The impact of such was also found in earlier research (Casamatta and Verb, 2000; Haefner *et al.,* 2004; Renke, 2010). If one were able to isolate such factors and understand how individually and collectively they affect the measures of the period of submersion, e.g. ADD, stage of decomposition and algal diversity, a more complete submersion timeline could produce a narrower range of the PMSI.

6.2.4 Shannon-Weiner Diversity Index as a Measure of PMSI

It is clear that the frequency of colonising taxa is an unpredictable determinant of the PMSI from the variability of abundance between sample sets and the absence of distinct trends. The absence of identifiable patterns and the problematic approach of using individual taxa mentioned earlier makes the abundance of colonising taxa alone impractical in estimating the PMSI. The absence of patterns found here were similarly found in earlier research (Casamatta and Verb, 2000; Zimmerman and Wallace, 2008).

The simultaneous analysis of abundance and diversity, represented by the Shannon-Weiner Index (H), however, may prove promising. The bimodal trend in H over the submersion period in both complete decomposition sample sets (winter 2018 and summer 2015) may prove a workable approach in determining the PMSI from algae. The degree of decomposition, however, will need to be considered in conjunction with H because H oscillates over time. The oscillation of H means that the absolute

value of H will relate to more than one submersion period. Differences in the absolute values of H between samples sets of this study emphasize the importance of factoring season and year when using H to determine the period of submersion. The advantages of using H as an PMSI indicator are that it includes more elements of the algal community (abundance, diversity, evenness) providing a more in-depth consideration of changes to the colonising community to derive the PMSI.

The bimodal trend in H seen in both seasons is understandable because, as diversity increases, the abundance of pioneering taxa, and successive colonisers, then intensifies competition for resources, e.g. space, nutrition and light (Keiper and Casamatta, 2001; Townsend *et al.,* 2008). The more competitive taxa dominate and result in an uneven spread of the remaining taxa (Townsend *et al.,* 2008), as indicated by the decrease in H (current study). The decrease in H in stage 3 in both sample sets is interesting as it indicates that decomposition, in particular the rupturing of the abdomen, affects the colonising community. This rupture event has been noted to have a negative impact on algal diversity and abundance before (Casamatta and Verb, 2000; Haefner *et al.,* 2004; Renke, 2010; Zimmerman and Wallace, 2008) and will be addressed further in section 6.1.7.2 below.

However, the drop in H may not be related to decomposition at all, as a similar decrease was seen on the glass microscope slides. The major oscillations found in H on the two substrates are not synchronised. This may be rooted in the lack of replication of samples and a resultant skewing of the H values of the colonising community. As a result, the effect of decomposition on H cannot be confirmed and this would need to be addressed in future studies.

In summary, analysis of algal diversity and abundance through the Shannon-Weiner Diversity Index, represents a more detailed approach when using algae to determine the PMSI. However, because the absolute value of H oscillates, it needs to be read in conjunction with the degree of decomposition of a body, so that an appropriate derivation of PMSI is made. Future research is needed to confirm this oscillatory response of H with progressive decomposition and thus determine whether this algal approach is indeed useful in estimating the PMSI.

6.2.5 The Effect of Decomposition on Algal Colonisation

The winter 2018 study was the first forensic study of algae as PMSI estimates to measure the change in pH and dissolved oxygen (DO). Where appropriate they will be considered in conjunction with the change of algae and decomposition over the submersion period.

6.2.5.1 Stage 1 and 2 (Fresh Stage and Bloated Stage)

The reduction in DO levels during stages 1 and 2 of winter 2018 was unexpected because elevated DO levels coincide with lower temperatures (Townsend *et al.,* 2008). This reduction therefore had to be in response to (a) factor/s other than temperature. One possibility is the increased metabolic demand of aerobic organisms as they colonise the carcass in these stages. A developing biofilm layer was seen at these earlier stages of decomposition and has been reported by others (e.g. Dickson *et al.,* 2011). The development of a biofilm, consisting of interacting organisms such as bacteria and fungi (Renke, 2010; Pechal and Benbow, 2016), increases resource competition, such as for DO (Renke, 2010). Such increased competition can negatively impact organisms (Townsend *et al*, 2008) such as algae colonising a body (Renke, 2010). All such factors that impact algal diversity and abundance will make the range PMSI determined from these measures broader. Colonising life forms other than algae, like bacteria (Pechal *et al*., 2013; Benbow *et al.,* 2015), may have potential for estimating the PMSI. . Analysis of the entire colonising community would place less emphasis on a single type of organism and offer a more complete picture of changes that occur on a carcass over the period of submersion.

6.2.5.2 Stage 3 (Floating Decay Stage)

The release of body fluid at the start of stage 3 occurred in close temporal-proximity with a decrease in the pH and dissolved oxygen (DO) of the water column and with fluctuations in the Shannon-Weiner Index (H) and algal diversity. Zimmerman and Wallace (2008) found a similar decrease in algal diversity with time but unlike the brief decrease in this study theirs was continuous. The transient dip in diversity is no doubt brought about by a change in the water column environment, such as the

observed drop in pH, caused by the introduction of the decompositional fluid following abdomen rupture. Such a drop and its negative impact on the algae colonising the carcass was also witnessed in a study by Casamatta and Verb (2000). The fluctuation in diversity and H may be related to the decreased DO levels brought on by the increased number of colonising organisms, such as bacteria decomposing the carcass. The number of colonising organisms increased due to the sudden availability of oxygen and the increased number of colonising organisms increased demand for oxygen. This resulted in the decreased DO levels seen in this study and potentially negatively impacted the colonising algae.

The change in algal growth in stage 3 may also be related to the impact of algal nutrient (Allan, 1995) that nutrient levels impact algal growth. Nutrients, particularly nitrogen and phosphorous, that exert an influence on algal growth (Allan and Castillo, 2007) are released during decomposition (Carter *et al.,* 2007; Killberg-Thorenson *et al.,* 2014). The concentrated nutrient levels, which become available shortly after abdominal rupturing, have a negative effect on the colonising algae (current study), as was found by Allan (1995) when he subjected algae to excessive quantities of nitrogen and phosphorus. The concentrated nutrient levels create an environment to which some taxa are intolerant (Allan, 1995; Townsend *et al.,* 2008) and explains the decrease in diversity and fluctuations of H seen in this study shortly after the abdomen ruptured. However, moderate levels of nutrients have a positive influence on algae (Fairchild *et al.,* 1985; Allan, 1995; Casamatta and Verb, 2000). This would explain the subsequent recovery in the algal measures of this study as the decompositional fluid mixed with the water and became diluted or used. The increase in algal diversity and abundance (current study), and likely that of aerobic bacteria (Dickson *et al.,* 2011; Benbow *et al.,* 2013), accounts for the decrease in DO at this time. Casamatta and Verb (2000) suggested the continuous increase in diversity in their study was due to the continuous release of nutrients during decomposition. As they did not measure algal abundance, the impact of nutrients released during decomposition was limited to algal diversity. The absence of a dip in algal diversity in their study, is felt to be rooted in their choice of analogue. Rats have smaller body masses and thus less influence on the water column, and hence colonising community, at rupture.

For algae to be useful in estimating PMSI, the influence of abdominal rupturing has to be considered. Although the effect on algal diversity was minor in this study, a greater volume of decompositional fluid that would be incurred with a decomposing human body will have a greater impact on the colonising algae. The effect of the release of decompositional fluid on algal colonisation should therefore be explored further. The response of algae to different concentrations of decompositional fluid should also be researched so this could can be taken into account when determining PMSI.

6.2.5.3 Stage 4 and 5 (Advanced Floating Decay Stage and Sunken Remains Stage)

The increase in diversity in early stage 4 (Advanced Decay Stage) can be explained with reference to the concept of disturbance ecology (Biggs and Smith, 2002; Townsend *et al., 2008*). Minor tissue loss exposed new uncolonized surfaces, resulting in a mix of early and late algal communities on the carcass and thereby an increase in algal diversity. However, when larger chunks of tissue detached later in decomposition, most of the established algae were lost and so the diversity was reduced to levels witnessed in early succession/colonisation. The absence in changes of diversity in previous work, (Casamatta and Verb, 2000; Zimmerman and Wallace, 2008) which was attributed to the progressive increase in tissue loss, is interesting. It is possible that the 31 day study period of Casamatta and Verb (2000) was sufficient to allow the rat carcasses to decompose but not for the effect of disturbance brought about by minor tissue loss on algal diversity (current study; Townsend *et al.,* 2008) to occur. The rapid colonisation and infrequent sampling in the 15 day study by Zimmerman and Wallace (2008) can explain the absence of fluctuations in diversity in their study as such changes fluctuations of diversity may have occurred on a non-sampling day.

It is therefore clear that tissue loss interferes with the estimation of PMSI via algal succession. The decline in algal diversity in the latter stages of succession, which was witnessed in communities on the glass microscope slides in this study, is altered as tissue detaches from the carcass. Additional research will be needed to determine whether any fluctuation in diversity as a result of tissue loss is predictable and will

allow the estimation of PMSI of a body that has reached the later stages of decomposition.

Clearly, the algal diversity found on a submerged body does not follow the expected successional dynamics but is impacted by the decomposition of the body. Algal colonisation is complicated by that of other organisms, by abdominal rupturing and by tissue loss. For algae to be used as a PMSI estimate, additional research will be needed to determine how decomposition itself affects the colonising community. Any trends that emerge can be taken into account when estimating the PMSI of a body that has reached these stages.

6.3 Algal Colonisation of the Glass Microscope Slides

6.3.1 The Effect of Season on Algal Colonisation of the Glass Microscope Slides

This was the first forensic study to consider the influence of season on the estimation of PMSI based on algae. Glass microscope slides were used to detect changes in the background noise of the colonising community between season and years, rather than to compare directly with those growing on the carcasses as recommended by Keiper and Casamatta (2001). Comparing the algae on two different substrates would offer no valuable information in advancing the use of algae as PMSI estimates as the colonising communities that would colonise each substrate would be different (Haefner *et al.,* 2004; Zimmerman and Wallace, 2008).

Season affects the rate of colonisation and community structure. Only after the winter substrates were colonised did the similarity (Sorenson's Similarity Index (C_s)) between seasons increase. However, the index never increased to a point where the algal communities across season were the same. The delay in colonisation in winter (current study; Renke, 2010), means that a PMSI determined from algal diversity or the dominance of various growth forms cannot readily be applied from one season to another, even for the same aquatic environment. Thus the effect of season on PMSI needs to be better investigated in future.

A comparison of the trends in algal colonisation (involving diversity, growth forms and Shannon-Weiner Diversity Index) in the same season, but in different years, shows that they are too variable to permit extrapolation from one year to any other. Thinking about this as a global application of algae as PMSI estimates of recovered bodies, it would be difficult to extrapolate the effect of more than one season on the algae colonising the body. It would be relatively straight forward to determine time from

algal measures made on the algal community for a body found in a location where seasonal variances are minor and the algal community remains somewhat consistent. It becomes increasingly complicated, however, trying to establish the relevant period of submersion for algal measures when the algae colonising the body has been influenced by the effect of more than one season.

Rainfall may be an issue in using algae to estimate the PMSI as it may account for the decrease in diversity seen on both summer substrates (glass microscope slides and carcass). Haefner *et al.* (2004) and Renke (2010) showed that rain events inhibited algal growth because of the increased physical disturbance of the water. Although the current study was conducted in a pond where there was no increase in physical disturbance through water flow, it is conceivable that a body can still be affected by rainfall if it is near the surface of the water and especially when the body is in the more advanced stages of decomposition. During the earlier stages of decomposition, the tissue of the carcass is still quite firmly attached and will unlikely be influenced by rainfall in a pond environment. However, as the tissue becomes more detached as decomposition advances, the increase in physical disturbance of the pond water brought on by rainfall will have more of an tissue loss and increase the exposure of uncolonized surfaces. The potential effect of rainfall increasing algal shearing and accelerating decomposition is thus a factor that should be considered when using algae as a PMSI. Haefner *et al.,* 2004 also recommended that this be considered in future research.

Both rainfall and temperature are useful means to determine environmental differences between seasons. They are major contributory factors (Haefner *et al.,* 2004) to the rate of decomposition of, and algal growth on, a body and can be determined through climatological data. Future research would be needed to confirm this and compare the validity of climatological data in this regard.

The analysis of algae colonising the glass microscope slides answered the objective that irrespective of decomposition, seasonality impacts algal growth. This means that the algae colonising the submerged carcasses will also experience the effect of seasonal difference regardless of decomposition. This complicates discerning the various factors that have resulted in the algae found colonising a body once it has been recovered. The difference in algal colonisation between season confirmed that seasons and year will need to be considered when using algae as PMSI estimate. Additional research would be needed to determine if and how major distinguishing factors between seasons, e.g. temperature and rainfall, can be adjusted for, collectively and individually, when trying to use algae to determine the period of submersion.

7 Limitations and Recommendations for Future Research

The constraints of the current investigation meant that only single ponds, analogues and samples could be monitored on each sample date. This is not ideal because it is impossible to know whether it is representative of a mean response. This can be overcome in future by the submersion of multiple analogues in multiple ponds and by taking multiple samples from each of these on each sample date. Increasing the number of sampling events would potentially offer a narrower range of the PMSI as it increases the number of replicas and so allows for statistical testing. It will, however, increase the disturbance of the carcass during sampling which might well impact on the colonising algal community (Biggs and Smith, 2002; Townsend *et al*., 2008). Increasing the number of analogues in a location could yield a narrower range of the period of submersion as outlying results and pattern in trends can be identified. The analogues could be sampled alternatively to reduce the sampling frequency for each individual carcass. The submersion of multiple analogues in a single body of water should be avoided as decomposition of these would excessively contaminate the water. To ensure this doesn't occur, regular readings of the water chemistry, e.g. DO, pH and nutrient levels, would need to be taken. The most ideal set up would be to submerge numerous analogues at once, each in their own pond of comparable volume, in the same location. This would offer true replication of samples and reduce the effect of disturbance. These constraints experienced in this study are very real constraints that will apply to future research as it cannot be guaranteed the number of carcass that will become available and challenging to find ponds of similar volume in the same location. The lack of replication prevents for statistical analysis of the data. Nevertheless, other research following and repeating this work in similar conditions may be able to

detect similar trends which can be used to narrow the range of PMSI or at least give a forensic scientist a better idea of the period of submersion of a recovered body.

The delay in decomposition in winter 2015 led to an incomplete data set for this season as the study was terminated after 42 days. This was corrected by conducting an additional winter study in 2018 in which the study continued until decomposition was complete. The additional study allowed for comparisons between season and year for the same season (winter) providing additional data useful in the application of algae as PMSI estimates. The pH and DO was measured in the winter 2018 study as additional abiotic factors that may be altered in response to decomposition, the increased number of colonising organisms on the carcass and the consideration of the effect of these factors on the colonising algal community. It is therefore recommended that future research include analysis of pH and DO regularly throughout the study.

A repeated disturbance brought about by the sampling strategy in studies such as this is likely to have implications. It will not only accelerate tissue loss from the carcass but will also impact the colonising algal community. With each removal of the carcass from the water, algae that are settled rather than attached (planktonic) will be dislodged. This in turn may affect the succession and diversity of the colonising community and therefore may skew any determination of PMSI. Repeated disturbance would also have an effect on the rate of decomposition of the carcass as the additional forces the carcass is exposed to will increase the rate of tissue loss from the carcass. To what extent such repeated disturbances impact on decomposition and colonisation, and their influence on the determination of PMSI, has yet to be confirmed.

The piglet in the winter 2015 was exposed to air on day 18 when the metal grid keeping the piglet submerged failed. Fortunately, this failure occurred

during a sampling event and exposure was limited to approximately five minutes. This period coincides with the approximate time taken when the carcass is exposed for algal sampling. As a result, the effects of this failure on the colonising algae was deemed negligible. Such a complication however needs to be considered in future research. Prolonged exposure of the carcass to the air will likely have negative effects on the algae colonising the carcass.

It is important to consider the conditions brought about by the two topping up events of the pond during the summer study and the effect this might have on the algae colonising the substrates. The opposing outcomes after each event (a decrease in benthic algal diversity after the first event and an increase in diversity after the second) indicated that changes in benthic diversity was unlikely to be related to the addition of water. As no discernible changes to the algae (diversity or abundance) colonising the substrates were noted in this study, and the low ratio of water added to the volume of the pond (0,94% of the total volume of the pond), it is unlikely that this impacted the colonising algae or the succession of algae. It is, however, possible that the addition of larger quantities of filtered water into the pond would change environmental conditions and in turn have an effect on the algae in the pond. It is therefore recommended that the addition of large quantities to the water be avoided in future studies.

The selection of the dorsal mid-line of the piglet as a sample site is considered a fortuitous strength of the present study (section 4.4.1) as this one of the last locations of the carcass to lose tissue. However, previous studies (Casmatta and Verb, 2000; Haefner *et al.,* 2004; Zimmerman and Wallace, 2008; Renke, 2010) were singularly silent on the selection of sample sites on the carcass and this makes direct comparison of the current study with theirs awkward, to say the least as the potential effect of decomposition, in particular exposure to decomposition fluid when the abdomen ruptures or tissue loss, cannot be taken into account. For

example, were samples collected from a distal part of a limb, they would not be exposed as much to any anticipated effects of the decompositional fluid and could be subject to much more sloughing of decomposing flesh that at the vertebral column. To offer a more systematic approach for comparison between studies it is recommended that future research include information about the origin of the samples, but the strong recommendation is that the vertebral column be adopted as a sensible site for taking the samples.

To advance the concept of using algae to estimate PMSI, it is recommended that future research should be conducted in the following areas:

i. Further research within forensic science with specific focus on decomposition should focus on defining universal, identifiable characteristics for classifying a submerged body into distinct stages of decomposition and the relative ADD for these stages. The inclusion of various aquatic environments in this process will allow consideration of distinct environment conditions, such as current, salinity, pH, and the effect they have on decomposition. A study establishing the temperature below which decomposition is arrested. This would allow the effect of temperature on the activity of decomposers acting on a body to be considered in future. A study could also be conducted to test the difference between recorded water temperature at the site and climatological data to trace back the temperature the carcass was exposed.

ii. The potentially useful measures of algae (diversity, growth forms and Shannon-Weiner Index) for estimation of the PMSI given in this research would all need to be reinforced through additional studies. The trends highlighted in each of these need to be scrutinised and confirmed. In addition, the impact of decomposition on each needs

to be addressed. Aspects of decomposition and how they influence algal colonisation should be studied further. For example, the effect of the release of decompositional fluid on the colonising algal community should be explored. In addition, the effect of tissue loss on the colonising community should be studied. The best sampling strategy to accommodate the uneven rate of tissue loss and facilitate comparison between studies should be studied so as to aid in the application of such algae as PMSI estimates to real life scenarios.

iii. The clear variation in decomposition and algal colonisation between seasons in this study highlights the need for more of this nature across season. It is recommended that the impact of rainfall and temperature, two major seasonal constituents, on decomposition and algal colonisation be investigated. Research should be conducted to determine if it is possible to incorporate and factor in climatological data into PMSI estimations.

iv. The issue with using algal growth forms to estimate PMSI is that it provides a threshold that is too broad to be useful. Future research should consider identifying more potential growth forms, such as forms that readily settle or those, other than filaments, that are attached.

v. It may be possible to link successional change in algae to ADD. This would provide a closer parallel with the use of arthropods in determining PMI in terrestrial environments and would take into account the difference of temperature between seasons.

vi. Future studies can include studies over different reasons allowing for the effect of seasons to be considered. Additional research in of the same season in different years can highlight any trends relating

to a particular seasons for a location. This will test the validity of the approach of algae as a PMSI determinant for all seasons and locations.

8 Conclusion

The presence of algae in all water habitats and the expected colonisation and succession of benthic algae on decomposing matter presents algae as a promising method of determining the PMSI. The main aim of this research was to assess the potential of algae as PMSI estimates in standing freshwater.

The following points have reference to the aim:

i. ADD (temperature and time) and the stage of decomposition alone were found in this study to be insufficient measures of the PMSI as these are too broad to offer any real indication of the period of submersion. Considering these with algal succession, however, is a promising approach to determine the PMSI.

ii. The TAD score derived from that proposed by Heaton *et al.,* (2008) was found to be an accurate method to estimate the PMSI. The estimated PMSI can be backtracked from the ADD calculated from an equation derived from the degree of decomposition (TADS) and the ADD. The difference in this equation between each of the three studies of the current study and compared with previous research (Heaton *et al.,* 2008, Humphreys *et al., 2013*) indicated that the relationship between ADD and TADS is influenced by environmental factors other than temperature. In order for an accurate PMSI to be determined from the equation developed from the ADD and TAD score, an equation would need to be determined for each scenario (location, season and year) the body was submersed. The degree of accuracy of the calculated ADD decreased with increasing PMSI and decomposition.

iii. Two approaches to determining the PMSI employing algae, viz. using changes in diversity and/or the form of growth over time, show promise. Additionally, the Shannon-Weiner Index (H), which considers diversity, abundance and evenness of algae, may be a useful indicator of the PMSI. The degree of decomposition would need to be considered with the absolute value of H to determine the PMSI as H oscillates over the period of submersion. The complexity of H would probably make any derived range of PMSI broader than desirable for the forensic scientist.

iv. This study was the first to consider the effect of season and year on the use of algae as PMSI determinants. It was also the first to consider the application of algae in determining the PMSI in South Africa making this thesis an important source of data for the emerging use of algae in forensic science in South Africa. A clear effect of seasons and variation of environmental factors that can occur from one year to the next for a season on algae was shown. Until such time that additional research can see if it is possible to even out the effect of season or years on algae as PMSI determinants, each scenario where algae is being used to determine the PMSI would have to be considered by itself and data cannot be readily applied to another scenario.

v. The effect of decomposition of the carcass on the colonising algal community was also highlighted in this research and it will therefore need to be considered when using algae to determine the PMSI of a body.

vi. Although the precise results of this study are only applicable to foetal bodies recovered for the particular aquatic environment in which the study was conducted, the concept of using algae as a PMSI determinant can be applied to any geographic location. More

studies are therefore needed to confirm the application of the algal measures given here and to consider these across seasons and in various locations.

This study shows there are measurable changes (Shannon-Weiner, diversity and presence of growth forms) of the algae colonising a submerged decomposing carcass over time. These measures have potential in being used to determine the PMSI of a carcass provided that season, the variation of the seasonal temperatures between years and the process of decomposition are accounted for.

9 References

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10 Appendix

Appendix 1

Ethics Approval

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/63/O

APPLICANT: Ms N Sloane

SCHOOL: Pathology
DEPARTMENT: Forensic Medicine
LOCATION: Medical School

PROJECT TITLE: Pig decomposition in freshwater rivers: the potential use of growing algae to establish the post-mortem submersion interval

Number and Species

4 piglets

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20141125. This approval remains valid until 20161124.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Warning notices to be erected along the water course
CAS to source and euthenase piglets
Weight range of piglets to be predetermined and reported by applicants
IBC approval may be required - please consult Iain.Burns@wits.ac.za
Logistics to be discussed in advance with Professor Candy

Signed: _____ (Chairperson, AESC) Date: 28th November 2014

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 28th November 2014

cc: Supervisor: Dr G Gordon
Director: CAS

Works 2000/Iain0015/AESCCert.wps

Appendix 2

September 2015 Rainfall record

LEGEND

The rainfall (in mm) as reported on the hour is the total of the rainfall reported over the previous hour

*** indicates that data is missing or is unavailable in the current month

HOURLY DATA : Rain(mm) - September 2015
JHB BOT TUINE - Climate Number:0475879 0 Lat:26°09'22"S Lon:27°59'56"E Height:1624 m (Extracted 2016/11/14 11:01)

DD	h01	h02	h03	h04	h05	h06	h07	h08	h09	h10	h11	h12	h13	h14	h15	h16	h17	h18	h19	h20	h21	h22	h23	h24	tot
1																									0,0
2																									0,0
3																									0,0
4																									0,0
5																									0,0
6																									0,0
7																									0,0
8																									0,0
9																									0,0
10																									0,0
11																									0,0
12																									0,0
13																									0,0
14																									0,0
15																									0,0
16																									0,0
17																									0,0
18																									0,0
19																									0,0
20																			2,2	25,6	7,4	1,4			36,6
21																									0,0
22																									0,0
23																									0,0
24																									0,0
25																									0,0
26																									0,0
27																									0,0
28																									0,0
29																								0,2	0,2
30	0,2																								0,2
tot																									37,0

Appendix 3

October 2015 Rainfall Record

HOURLY DATA : Rain(mm) - October 2015																									
JHB BOT TUINE - Climate Number:0475879 0 Lat:26°09'22"S Lon:27°59'56"E Height:1624 m (Extracted 2016/11/14 11:01)																									
DD	h01	h02	h03	h04	h05	h06	h07	h08	h09	h10	h11	h12	h13	h14	h15	h16	h17	h18	h19	h20	h21	h22	h23	h24	Tot
1																									0,0
2																									0,0
3																									0,0
4																									0,0
5																									0,0
6																									0,0
7																									0,0
8								19																	0,0
9																									0,0
10																		6,0							6,0
11																									0,0
12																									0,0
13																									0,0
14																									0,0
15																0,2	0,2	0,4							0,8
16			0,2																						0,2
17																									0,0
18																									0,0
19																									0,0
20																	0,2								0,2
21							0,2		0,6				0,2				0,6	0,6	0,4						2,6
22							0,2											4,2							4,4
23																		7,6	9,8	1,2					18,6
24		1,6	0,2	0,2	0,4	0,2	1,2	4,8									1,0	1,4							11,0
25																					12,0	7,8	2,6	1,2	23,6
26	0,4			0,8																					1,2
27																									0,0
28																									0,0
29																									0,0
30																									0,0
31																									0,0
tot																									68,6

Appendix 4

November 2015 Rainfall Record

HOURLY DATA : Rain(mm) - November 2015
JHB BOT TUINE - Climate Number:0475879 0 Lat:26°09'22"S Lon:27°59'56"E Height:1624 m (Extracted 2016/11/14 11:01)

DD	h01	h02	h03	h04	h05	h06	h07	h08	h09	h10	h11	h12	h13	h14	h15	h16	h17	h18	h19	h20	h21	h22	h23	h24	tot
1																									0,0
2						2,8	12,0	7,8	2,6	1,2															26,4
3														5,8	8,2	4,8	3,2	2,6	1,4	1,2					27,2
4										1,8	5,0				0,2	0,2									7,2
5																									0,0
6																									0,0
7																									0,0
8																0,2	1,0	0,6							1,8
9							3,2	0,2	0,2				0,2					0,2		1,2	0,8				6,0
10										1,6						0,4	0,8	0,2	0,4			0,2			3,6
11		4,2	1,6	0,4				0,4		1,6	3,2	4,2	0,8	2,2	1,4	4,6	1,0	0,6			0,4				26,6
12					1,4		0,2	1,8										0,6	0,4			1,2	0,2		5,8
13	0,2																								0,2
14																									0,0
15									6,2	0,2	0,6						7,2	0,2							14,4
16																									0,0
17													11,6	7,6				0,2							19,4
18																									0,0
19															0,8										0,8
20															0,4										0,4
21																0,2									0,2
22																									0,0
23									1,2	7,4	1,6	2,6	1,6												14,4
24															2,0		1,0	2,4	3,4		0,4	0,4			9,6
25		0,4								0,2															0,6
26																								10,4	10,4
27	3,0	6,2	5,6	1,8	0,2																				16,8
28																									0,0
29																									0,0
30																2,2	4,8								7,0
tot																									198,8

Appendix 5

Decomposition Record Sheet

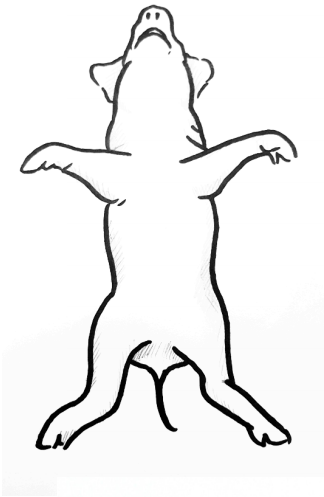
RECORD OF PHYSICAL CHANGES OF THE SUBMERGED PIGLET IN FRESHWATER POND

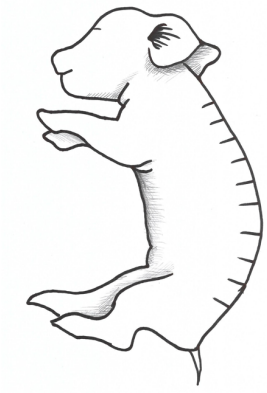
Date :

Season: Summer Winter

Day Number:

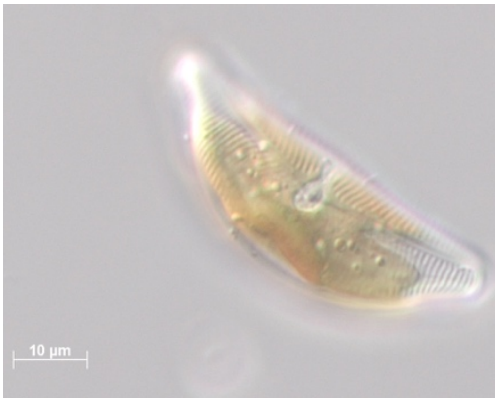
Appearance of Pig:





Appendix 6

Micrographs of Some of the Colonising Algal Taxa



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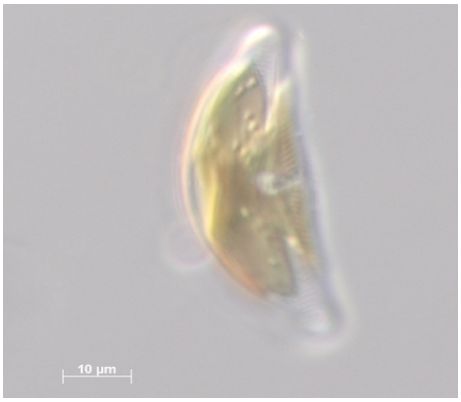

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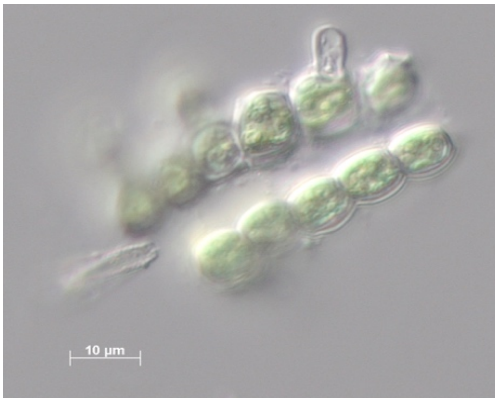

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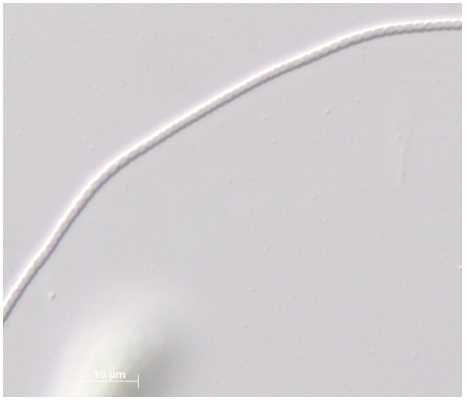

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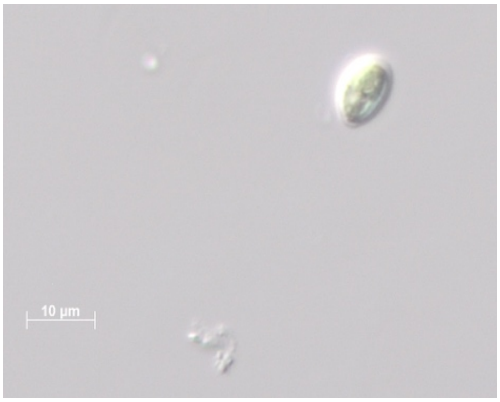

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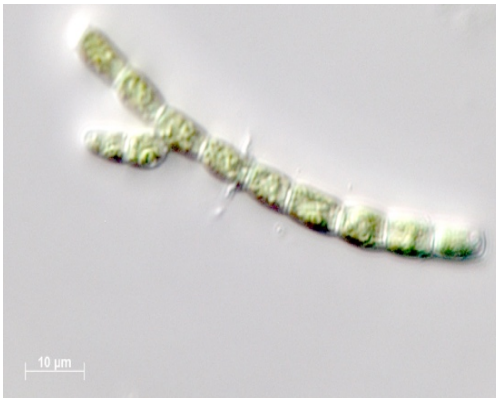
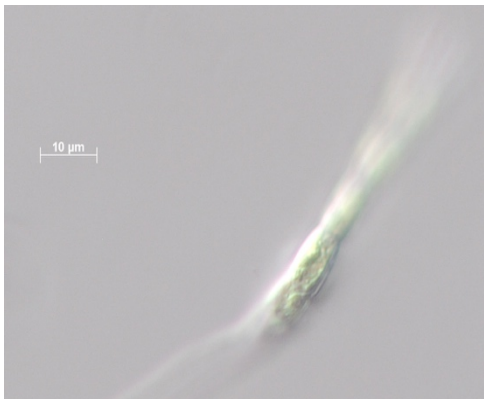


6)

1) Amphora 2) *Amphora* 3) *Coleochaete* 4) *Anabaena*

5) *Chlamydomonas* 6) *Coleochaete*



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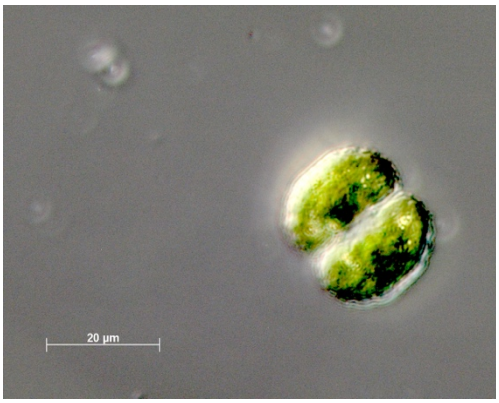

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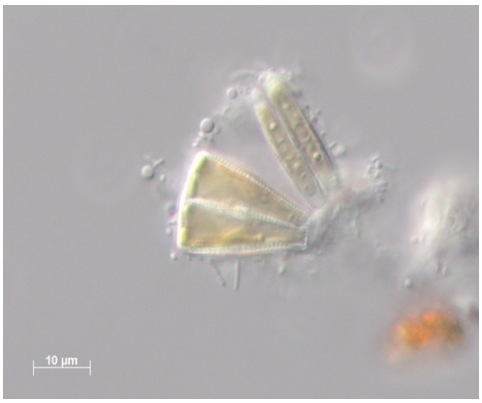

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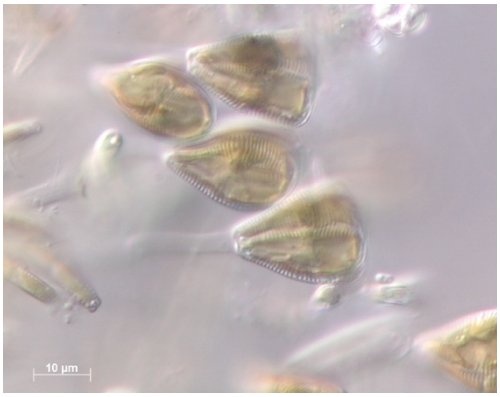

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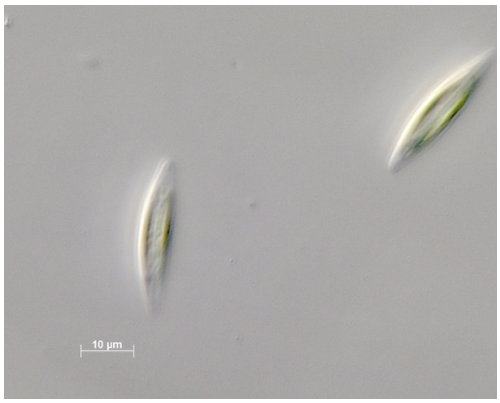

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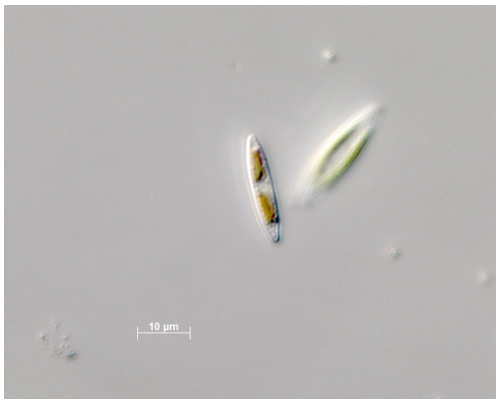
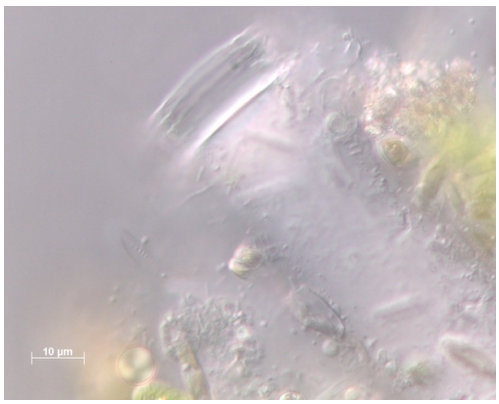


12)

7) *Coleochaete* 8) *Cosmarium* 9) *Gomphonema* 10) *Gomphonema* (note stalks for attachment)11) *Navicula* 12) *Nitzschia* (left) and *Navicula*



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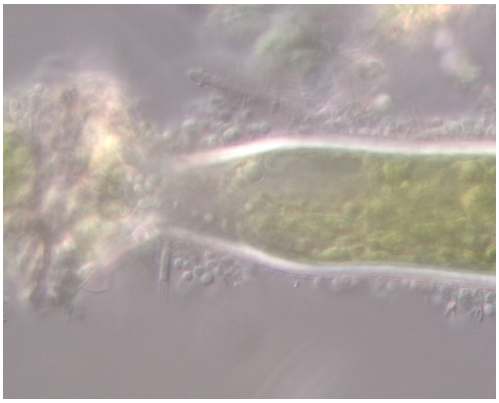

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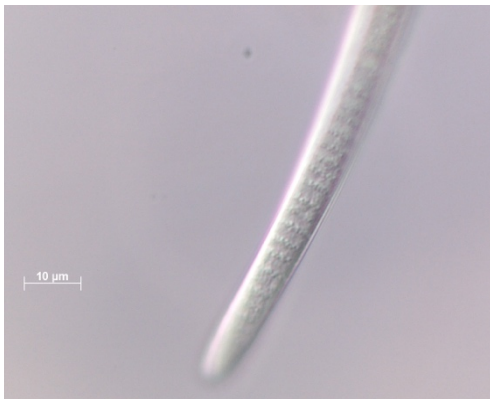

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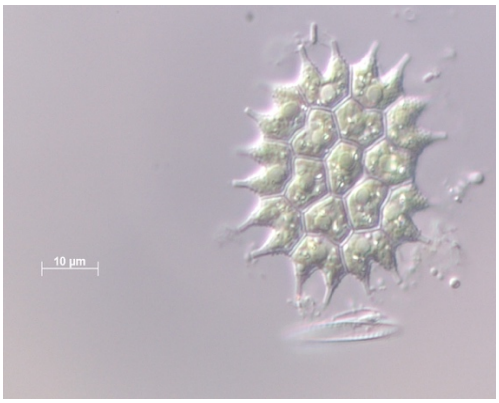

16)

13) Cap cells of *Oedogonium* 14) Attachment of *Oedogonium* 15) *Oscillatoria* filament 16) *Pediastrum*

Appendix 7

Descriptive stages for decomposition observed in the face and the assigned facial aquatic decompositional score (FADS) (from Heaton et al. (2010)).

1 pt	No visible changes.
2 pt	Slight pink discoloration, darkened lips, goose pimpling.
3 pt	Reddening of face and neck, marbling visible on face, Possible early signs of animal activity/predation-concentrated on the ears, nose, and lips.
4 pt	Bloating of the face, green discoloration, skin beginning to slough off.
5 pt	Head hair beginning to slough off-mostly at the front. Brain softening and becoming liquefied. Tissue becoming exposed on face and neck. Green/black discoloration.
6 pt	Bone becoming exposed-concentrated over the orbital , frontal, and parietal regions. Some on the mandible and maxilla. Early adipocere formation.
7 pt	More extensive skeletization on the cranium. Disarticulation of mandible.
8 pt	Complete disarticulation of the skull from torso. Extensive adipocere formation.

Descriptive stages for decomposition observed on the torso and the assigned body aquatic decompositional score (BADS) (from Heaton et al. (2010)).

1 pt	No visible changes.
2 pt	Slight pink discoloration, goose pimpling.
3 pt	Yellow/green discoloration of the abdomen and upper chest. Marbling. Internal organs beginning to decompose/autolysis.
4 pt	Dark green discoloration of abdomen, mild bloating of abdomen, initial skin slippage.
5 pt	Green/purple discoloration, extensive abdominal bloating-tense to touch, swollen scrotum in males, exposure of underlying fat and tissues.
6 pt	Black discoloration, bloating becoming softer, initial exposure of internal organs and bones.
7 pt	Further loss of tissues and organs, more bone exposed, initial adipocere formation.
8 pt	Complete skeletonisation and disarticulation.

Descriptive stages for decomposition observed in the limbs and the assigned limb aquatic decompositional score (LADS) (from Heaton et al. (2010)).

1 pt	No visible changes.
2 pt	Mild wrinkling of skin on hands and/or feet. Possible goose pimpling.
3 pt	Skin on palms of hands and/or soles of feet becoming white, wrinkled, and thickened. Slight pink discoloration of arms and legs.
4 pt	Skin on palms of hands and/or soles of feet becoming soggy and loose. Marbling of the limbs-predominantly on upper arms and legs.
5 pt	Skin on hands/feet starting to slough off. Yellow/green to green/black discoloration on arms and/or legs. Initial skin slippage on arms and/or legs.
6 pt	Degloving of hands and/or feet-exposing large areas of underlying muscles and tendons. Patchy sloughing of skin on arms and/or legs.
7 pt	Exposure of bones of hands and/or feet. Muscles, tendons, and small areas of bone exposed in lower arms and/or legs.
8 pt	Bones of hands and/or feet beginning to disarticulate. Bones of upper arms and/or legs becoming exposed.
9 pt	Complete skeletonization and disarticulation of limbs.

Appendix 8

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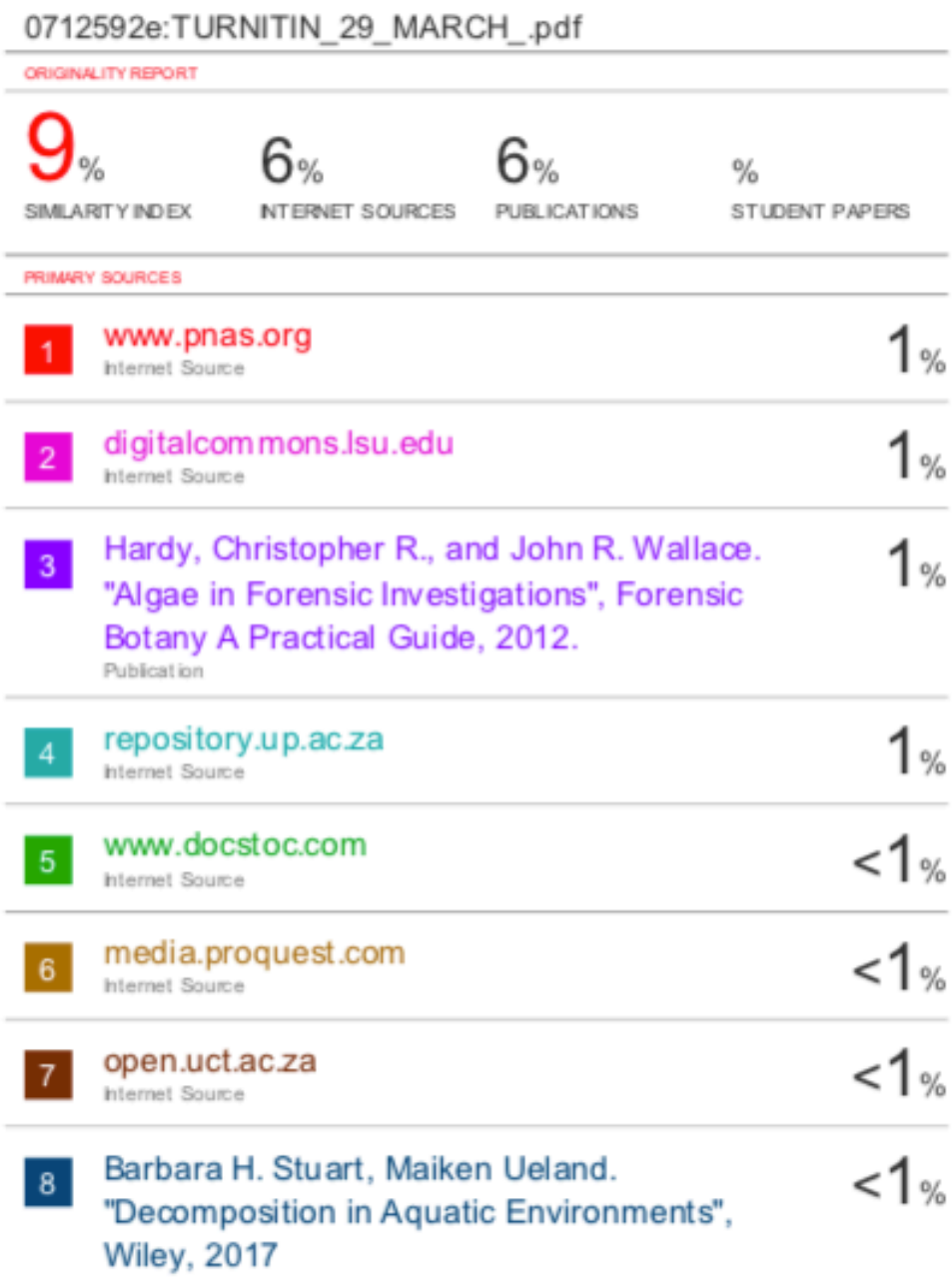

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