A Study on the Phototrophic Microbial Mat Communities of Sulphur Mountain Thermal Springs and their Association with the Endangered, Endemic Snail *Physella johnsoni*

By

Michael Bilyj

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Abstract

The seasonal population fluctuation of anoxygenic phototrophs and the diversity of cyanobacteria at the Sulphur Mountain thermal springs of Banff, Canada were investigated and compared to the drastic population changes of the endangered snail *Physella johnsoni*. A new species and two strains of *Rhodomicrobium* were taxonomically characterized in addition to new species of *Rhodobacter* and *Erythromicrobium*. Major mat-forming organisms included *Thiothrix*-like species, oxygenic phototrophs of genera *Spirulina*, *Oscillatoria*, and *Phormidium* and purple nonsulfur bacteria *Rhodobacter*, *Rhodopseudomonas* and *Rhodomicrobium*. Aerobic anoxygenic phototrophs comprised upwards of 9.6×10^4 CFU/cm^2 of mat or 18.9% of total aerobic heterotrophic bacterial isolates at certain sites, while maximal purple nonsulfur and purple sulfur bacteria were quantified at 3.2×10^5 and 2.0×10^6 CFU/cm^2 of mat, respectively. Photosynthetic activity measurements revealed incredibly productive carbon fixation rates averaging 40.5 mg C/cm^2/24 h. A temporal mismatch was observed for mat area and prokaryote-based organics to *P. johnsoni* population flux in a "tracking inertia" manner.

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1.1.1 Sulphur Mountain and Thermal Springs

Sulphur Mountain lies in the front ranges of the southern Canadian Cordillera, a thrust dating back to the massive collision of oceanic arcs (underwater islands created by the subduction of tectonic plates) with the western coast of North America during the Jurassic to Tertiary periods, roughly 135 to 63 million years ago (Mya) (Grasby & Hutcheon, 2001). As with the surrounding Rundle, Bourgeau and McConnell thrusts, Sulphur is generally comprised of Paleozoic carbonates forming hundreds of kilometer-long ranges. Sulphur is somewhat distinct in the expression of its hydrogeology with nine (compared to Bourgeau's one and Rundle's two) calcium-rich thermal springs clustered near the town of Banff, Alberta, one of which was instrumental in the creation of the Banff National Park itself (Grasby & Lepitzki, 2002). What defines these freshwater thermal springs? Specifics of Sulphur Mountain water chemistry will be discussed in Subsection 1.1.2, but in general these springs can be considered as harsh environments with relatively high temperatures, elevated concentrations of dissolved minerals and low dissolved oxygen (Lepitzki, 2002). The springs are fed by meteoric waters (rain and snowfall) that have infiltrated cracks in the rocks of local mountains through what is known as recharge zones (Grasby & Lepitzki, 2002). Water is heated by the earth's thermal gradient, resurfacing along the Sulphur Mountain Thrust faults that crosscut the actual thrust sheet of rock. In short, these discharge faults are believed to be the results of the force Mount Rundle's underlying transverse rock layers (from sea level

to below 2000 m) apply towards the Sulphur Mountain Thrust. Estimated minimum water circulation depth is taken at 3.2 ± 0.64 km from the source (Grasby & Lepitzki, 2002).

The slightly ambiguous descriptor *thermal* should also be addressed. According to Canadian Oxford Dictionary (2004), thermal describes "of, for or producing heat", though to what degree it is uncertain. Often the anthropocentric guideline of average body temperature (36.7 °C) is used to distinguish between hot, warm and cool springs. Due to the larger range of possibilities above body temperature than below and the relativity in terms of how different observers would experience what is "hot" or "warm", it makes this approach an imperfect benchmark. Pentecost *et al* (2003) published the collective responses of researchers to a questionnaire on the proper usage of the terms hot, thermal, warm and cold in relation to springs. While the possibilities for definitions were not exhaustive, respondents were split by the proposed definition for thermal to reflect pools where "mean water temperature higher than mean air temperature". Aside from other difficulties, in these terms even a frigid spring could be described as thermal in Banff where annual average air temperature is about –0.4 °C (Grasby & Lepitzki, 2002). All such prefixes continue to be used in the literature and without the invention of new terms such as ambient or *super* ambient as suggested in a later Pentecost article (2005), the present regional pluralism will have to suffice. For the purposes of this project and all the work done by other scientists at Sulphur Mountain, *thermal* is used consistently to refer to those springs of temperature relatively near body temperature and "cool" was used for one spring where the maximum recorded temperature was 19.6 °C (Lepitzki, 2007).

1.1.2 Human Activity and Historic Spring Sites

Human activity in the Bow Valley dates back to over 10000 years to the Nakoda or "Stoney" First Nation group of people, linguistically related to the Dakota and Lakota of the large Sioux Nation (Dmytriev, 1997). They attributed spiritual and healing properties to the springs on Sulphur Mountain's slopes, but in time it was three Canadian Pacific Railway workers that brought capital and enterprise to these mineral waters.

It was 1871 when, in fulfillment of a government promise to link British Columbia with the other four Canadian provinces, the building of our national railway began (TOB, 2007). In the fall of 1883 rail workers William McCardell, his brother Tom and partner Frank McCabe discovered a 6 meter warm, water-filled cave. The discovery of the "Cave Spring" led to staking claim and opening a bathing resort, though arguments over ownership led to Canadian government involvement to settle the dispute (TOB, 2007). The opportunity for federal prestige and the promotion of the area (as an international resort and spa to support the new railway and ease the financial pressures on Confederation) was duly seized and intentions to nationalize the area were declared in 1885, thus making the Cave Spring the epicenter of Canada's first National Park. In that same year, former CPR director Lord Steven christened the area. Human influx, activity and development has not ceased since this time.

In the 1970's and 1980's Van Everdingen pioneered studies focused on the seasonal variations of Sulphur Mountain springs, describing that "the natural conditions in the area have been modified irrevocably by the construction of the existing facilities…It is thus impossible to return the area to its natural state" (Van Everdingen &

Banner, 1982). The Cave and Basin now exists as a Historic Tourist Site consisting of three buildings and two boardwalks guiding observers past four separate springs: Cave, Basin, Upper and Lower. The multitude of tourists that visit the site annually easily exceeds 100000, for example nearly 165000 during the 1998/1999 fiscal year (Lepitzki *et al.*, 2002).

By contrast, a second group of Sulphur Mountain springs is the least affected by human activity and exist close to their original state. The Upper and Lower Middle springs are just less than one kilometer straight-line distance from the Cave and Basin and 100 m higher (at 1500 m) in elevation. They are fed by three sources and are generally less accessible, but it is believed the volume and course of outflow nevertheless were affected by human activity. Two examples include fulfilling the needs of Dr. Brett's Sanatorium and his *Banff Lithia Water,* 1886-1888, and water piping to the Cave and Basin in the 1950's (Lepitzki *et al.*, 2002). In 1988, Environment Canada designated the spring area an Environmentally Sensitive Site for the preservation of the proximal wildlife corridor and the habitat that hosts rare plants and invertebrates (Lepitzki *et al.*, 2002). Following a human fecal pollution incident in 1995, the area was officially closed to human presence by Superintendent's order in 1997 and at present exists as an electronically monitored, routinely patrolled area accessible only to wildlife and permit holders (Lepitzki *et al.*, 2002).

The last two springs with origins on Sulphur Mountain are the Upper Hot Spring and Kidney Spring, though the project at hand involved neither of these. The Upper Hot Spring presently exists as the only commercially developed spring in Banff National Park (the Banff Springs Hotel), displacing most indigenous fauna with almost three-quarters of

a million bathers per year. Clarke (1973) recorded collection of *Physella johnsoni* from an area about 300 m south of the Banff Springs Hotel, but this area has since undergone irrevocable changes with trails, landscaping and construction (Lepitzki *et al.*, 2002). Secondly, while Clarke (1973) also recorded Kidney spring as a collection site of *P. johnsoni*, less than 10 shells have ever been found there until the successful reintroduction of 50 snails in November 2003 (Lepitzki, 2007).

1.1.3 Climate and Spring Water Characteristics

A combination of northern latitude (N51° 10'), immediate proximity to the mountains of the Continental Divide and prevailing westerly winds create Banff's ever-variable weather conditions. Zephyrs bring moist air from the Pacific Ocean to Banff consequently being forced up and over British Columbia's mountain peaks. The greatest obstacle is the Continental Divide forming the natural border between the two provinces, cooling the air and dumping precipitation on the windward slopes; relatively low humidity is thus one general feature of Banff National Park weather. Accumulated annual precipitation is near 280 mm rainfall, but quite variable (e.g. 381 mm of rainfall and 240 cm snowfall occurred in 2008 (NCDIA, 2005). This is not unlike the Yellowstone National Park area springs that experience nearly the same average precipitation accumulation (380.2 mm), but at an annual average of 180.1 cm of snow (WRCC, 2005). For comparison, our own Winnipeg prairie climate had 627 mm of rainfall in 2008 and an average just over 110 cm snowfall (NCDIA, 2005).

The warmest and coldest months in Banff National Park are June and January with average highs around +22 °C and lows near –15 °C, respectively (Parks, 2009).

With latitude and location among the surrounding mountain peaks, the sun falls on Banff at angles that lessen its warming power.

Climate influences everything in the park and despite the apparent physico-chemical stability of a hot or thermal spring ecosystem, the seasonal diel cycle and influx of spring melt water are two of the more important regularly-varying features. Length of day varies appreciably in Banff from its average of 16.5 h in June to less than half that value in December, 7.8 h (Parks 2009). This is quite significant for the light-capturing phototrophic bacteria (Section 1.3), which form one of the most notable and important features within the spring streams and pools.

As spring discharge depends on meteoric water, an annual pattern of reduced infiltration of rain and snow in the winter/early spring and increased flow in late spring emerges. The Upper Hot Springs went dry for two months between March and May 1923 (following the second lowest recorded total annual accumulated precipitation) and a 70% drop in flow rate occurred in 1970 after the third lowest accumulated precipitation value of 325 mm (Grasby & Lepitzki, 2002). The springs reach their maximum temperatures during the winter when infiltration is reduced. During the spring melt, additional shallow ground water results in elevated flow rate, pH and dissolved oxygen and decreased water temperature, conductivity and sulfide levels (Lepitzki, 2007). Springs at lower elevations have generally demonstrated the lowest seasonal variation and highest levels in total dissolved solids (TDS). For example, the Basin averaged 2030 mg/l compared to 1070 mg/l at Kidney Springs, while higher-elevation springs have shown TDS drops of one half from winter to summer. Major ion concentrations in these springs include on average (mg/l): Ca^{2+} ranging from 240 at the Lower Middle Springs to

414 in the Basin pool, Mg^{2+} from 37 at Kidney to 75.6 at the Basin and Na^{+} from 5.5 at the Upper Middle to 7.1 at the Basin. While there is variation between individual springs, ion concentrations within each do remain relatively constant throughout the year and rather significant variation occurs in HCO_3^- and SO_4^{2-} as a function of TDS. SO_4^{2-}/HCO_3^- ratios were observed to vary linearly with TDS. This relationship was explained as "a geochemical evolution of groundwater as a function of residence time along flow path" rather than simple mixing and dilution of SO_4-rich, high-TDS thermal water with cooler HCO_3^--rich groundwater, since the highest temperature spring (Upper Hot at 41 °C) also had the lowest TDS. On average concentrations of HCO_3^- range between 126 and 180 mg/l for all springs, while SO_4^{2-} is near 700 mg/l for every spring except the Basin (1330 mg/l). Sulfide has the most variability between springs, from 1.7 mg/l at Kidney, to 14.7 mg/l in the Basin, 36.7 mg/l at the Upper Middle and 45.7 mg/l within the Cave (Lepitzki, 2002).

1.1.4 Scientific Interest

Interest in the springs extends beyond the history of human use and touristic potential to Banff's biodiversity and unexplored microbiology. Section 1.3 expands on the most prominent feature of the springs: the laminated microbial mats present within stream outflows or often attached to detrital matter floating atop pools. The combination of temperate water, abundant electron donors, illumination and constant supply of microelements contribute to the proliferation of bacterial and microscopic eukaryotic assemblages. The study of these mats will not only enrich understanding of the microbial diversity in the environment, but help reveal fundamental principles of community ecology. As Kilian *et al.* (2007) stated, spring communities represent excellent model

systems for exploring how environmental parameters such as light intensity, temperature, nutrient availability and oxygen levels shape the structural and fundamental aspects of the community. These mats simultaneously form the parts of the basal and secondary trophic levels of the ecosystem as a complex and tightly knit assembly of primary producers and heterotrophic decomposers (Hall & Meyer, 1998; Overmann & Garcia-Pichel, 2006).

Regarding biodiversity within Banff National Park, Section 1.2 explores in depth the endangered, endemic snail *Physella johnsoni*, which resides at only a few locations along the spring streams of Sulphur Mountain and has garnered great attention from conservationists over the last 14 years (Grasby & Lepitzki, 2002; Lepitzki, 2002).

1.2 Physella johnsoni, the Banff Springs Snail

1.2.1 Species Description

Phylum Mollusca is divided into 6 classes including Gastropoda, comprising almost 80% of the estimated near 80000 species within the 409 families of the phylum (Bouchet *et al.*, 2005). Fossils date molluscan origins to the Cambrian period over 500 Mya making them one of the most ancient animal groups (Clarke, 1973), for example, predating sharks by over 100 My. As shelled aquatic invertebrates, geographic distribution of Gastropoda is influenced by two fundamentals: waterway linkages and access to calcareous sediments and materials (e.g. limestone) for shell growth. For example, bedrock in the Cordillera region is of Pleistocene orogenic origin (2 Mya to 10000 years ago (ya), related to the formation of mountains) and contains such calcium-rich mineral deposits (Clarke, 1973). Distribution of gastropods in the Canadian Interior Basin is also linked to

fauna surviving in ice-free zones during the four glacial periods in North America: Nebraskan, 1.2 Mya; Kansan, 470000 ya; Illinoian, 200000 ya and Wisconsian, 115000 ya. In the broadest terms, confluence and drainage of ancient glacial Lake Agassiz (existing ~12000 to 7000 ya) and, in the east, Lake Barlow-Ojibway (~8000 to 6000 ya) also influenced the present distribution of gastropods in Canada (Clarke, 1973).

Physella johnsoni (Fig. 1) was first described by Clench in 1926, who named the species in honor of the entomologist that sent the material for species determination (Lepitzki *et al.*, 2002). *Physa* were separated from *Physella* by an overlapping mantle (the organ that forms the dorsal surface body wall, secretes the shell and, in pulmonates, encloses the lung cavity) with digitations on both sides in the former genus and only behind the head in the latter (Lepitzki *et al.*, 2002). While Baker commented in the mid-1920's that *Physa* might have been improperly extended to other physids with respect to mantle traits, it wasn't for another 60 years that Te (1978) finally confirmed these observations in *P. johnsoni* (Lepitzki *et al.*, 2002). Using 37 shell and 34 anatomical characteristics, including mantle and penial complex morphology in a numerical taxonomic approach, the species was assigned to *Physella*. Like other species of the family Physidae, one of *P. johnsoni*'*s* most distinguishing traits is a shell that coils sinistrally meaning, when the shell apex is upward, the opening/aperture is on the left (Lepitzki *et al.*, 2002). Its size is also notable, with shell length averaging around only 5 mm long, though shells up to 11 mm have been found (Lepitzki, 2002).

Fig. 1. The Banff Springs snail, *Physella johnsoni*. Bar, 5 mm.

Presently, morphological characteristics in concert with allozyme analysis (e.g. cytochrome *c* oxidase sequencing) and mitochondrial sequencing (of the 16S rRNA subunit gene) are used for species description and determination of evolutionary lineage (Remigio *et al.*, 2001). Using such sequence variation analysis, phylogenetic reconstructions by Remigio *et al.* (2001) suggested that *Physella wrighti* might have been the source of the ancestral population from which *P. johnsoni* and subfamily relative *P. gyrina* were derived. They were also able to link the low level of mtDNA sequence divergence between *P. johnsoni* and *P. gyrina* to the recent age of the Banff habitats, which likely originated (or reestablished) only 10000 ya, when glaciers retreated from this region (Pielou, 1991). The separation of the two species most probably occurred at this time as ancestral populations of *P. gyrina* adapted to life in the newly arisen thermal pools and diverged genetically to produce *P. johnsoni* (Remigio *et al.*, 2001). Such a recent origin could make *P. johnsoni* a model for evolutionary studies (Lepitzki *et al.*, 2002).

As of twelve years ago, there were still no published data on the reproductive biology, movement, behavior or ecology of *P. johnsoni* and most subsequent findings are contained in Parks Canada reports first prepared in 1997 (Lepitzki *et al.*, 2002). Regarding reproductive biology, *P. johnsoni* is most likely hermaphroditic. Whereas pulmonates are typically annual, breeding once and then dying with one new generation per year (semelparous), increased temperatures can accelerate reproduction and lead to more than one generation annually (Lepitzki, 2002). There also appears to be a size threshold that is influenced by nutrient availability and predation. Studies on *Physella virgata* (Crowl & Covich, 1990) discovered that snails grew to about 4 mm shell length (~3.5 months) before beginning reproduction, but in the presence of predator stress, exhibited rapid growth rates and low reproduction rates until they reached about 10 mm in length after about 8 months. Any size threshold in *P. johnsoni* is yet unknown. Documented in *P. virgata* (Britton & McMahon, 2004) is a type of temperature-dependent fecundity, where assimilation of organic carbon was stable at 25-35% efficiency within their natural temperature range (15-35 °C), but fell to <10% when temperatures were artificially elevated to 40 °C. This may or may not be influential for *P. johnsoni*, as very small (~1 mm shell length) snails and egg capsules are observed year round, and are omnipresent at or slightly above the water surface attached to any hard substrate including pool walls, wooden posts or the microbial mat (Lepitzki, 2002).

1.2.2 Distribution, Population Trends and Limiting Factors

P. johnsoni is endemic to the springs at Sulphur Mountain. While there is some uncertainty in exact dates, its distribution until the 1920's included the Cave, the Cave and Basin Lower and Upper springs, the Middle Springs, the Upper Hot Springs, Kidney

Spring and the cool springs at Vermillion Lake, (3 km from the Cave and Basin and having a different origin than those on Sulphur Mountain) (Lepitzki, 2002). Snails only remained at the Lower Middle spring, and the four springs at the Cave and Basin until translocation of fifty Lower Middle specimens to the Upper Middle in November 2002 and one year later, the transfer of fifty and twenty-five each from the Upper and Lower Middle, to establish a Kidney Springs population (Lepitzki, 2007). The re-establishment of these latter two populations was executed as part of the COSEWIC-mandated Research and Recovery Program discussed in the following subsection. The entire critical habitat of the snail presently encompasses a mere 170 m^2 within Banff National Park (Lepitzki, 2002). All springs have been affected by human activity in some way and while security measures have been taken, interference still occurs. Kidney Springs fall outside any Superintendent closure and are frequented by people who enjoy soaking in the concrete cistern (Lepitzki, 2002). Even the extensive signage at the Cave and Basin Historic Tourist site cannot eliminate all accidental hand or foot immersion into the waters or deter intentional vandalism. For example in April 2007, culprits bypassed the security fence to swim in the Basin, scattering the microbial mat and inadvertently killing several snails (CanWest, 2007). Habitat alteration can also come from simple acts of littering with garbage or coins (copper sulfate was once used as a molluscicide), kicking of snow and ice or removal/movement of rocks or twigs (Lepitzki, 2002). Natural mortality also results from at least three sources including potential predation by waterfowl and garter snakes and the impact of disease and parasites. A phenomenon called “twitch-ups” is where snow-laden branches are bowed near enough to streams that bacteria and snails colonize them until melting releases the branch in a twitching motion

that flings or leaves stranded any attached snails to bear winter temperatures reaching far below freezing (Lepitzki, 2002).

Populations within each thermal spring typically fluctuate by over two orders of magnitude annually with lows in late spring/early summer (May-July) and peaks in late fall/mid-winter (November-February) (Lepitzki, 2007). These extreme fluctuations occur even with limited anthropogenic interference; i.e. similar patterns are observed at the restricted zone of the Middle Springs and the heavy traffic Cave and Basin area. Such drastic fluctuation patterns are not unheard of in other species of aquatic snails. For example, it was observed that *Biomphalaria pfeifferi* in Zaire dropped in annual density by over 80% (Loreau *et al.*, 1987) and in a 3-year study *Cepaea vindobonensis* in northern Greece was observed to vary from 49% to 64% annually (Staikou, 1998). Several other studies assessing population density have also demonstrated natural fluctuations upwards of 90% (Chingwena *et al.*, 2004; Dana & Appleton, 2007; Mouthon, 2007; Yapi *et al.*, 1994). Combined with high risk factors of human activity, spring water dry-outs, small habitat and range, these severe annual fluctuations contributed greatly to the endangered status of the Banff Springs snail. The extirpation of *P. johnsoni* from two of its original locations spurred a 1996 population data and distribution study that was compiled into a COSEWIC Status Report and the beginnings of a Recovery Plan (Lepitzki, 2002). By 1998, a second draft of the multi-faceted "Recovery Plan for the Banff Springs Snail" was developed to include water physico-chemical analysis. The final 2002 draft includes the fundamental goal stated as the re-establishment of "self-sustaining populations at the Upper Middle and Kidney Springs while maintaining and enhancing present populations" in concert with the collection of environmental

parameters (Lepitzki *et al.*, 2002). The population and water chemistry survey culminated in a recent "10-year-plus data summary for the Banff Springs Snail" by Lepitzki (2007). As mentioned above, the first objective of re-establishment has been successful, but elucidating the key factors and parameters within this limited critical habitat, including the microbiological aspect, is ongoing.

1.2.3 Significance and Recovery Management

"Just as large carnivores such as grizzly bears are used to indicate the ecological integrity or health of large ecosystems such as the Eastern Slopes of the Canadian Rockies, the health of the thermal spring ecosystems on Sulphur Mountain can be determined by the health of their most prominent inhabitant- the Banff Springs Snail" (Lepitzki, 2002).

The Banff Springs snail may thus be the biological equivalent of a thermometer or the proverbial "canary in the coal mine". Indicator species are responders to habitat change and can serve as models to study environmental cause-and-effect, which can in turn contribute to ecologists' predictive capabilities when studying other similar biomes. *P. johnsoni* may also play a role at the opposite end of the "arrow of influence" as a catalyst of habitat change or an instrument in maintaining habitat integrity, or community in the form which it exists. A species with such a disproportionate effect on its environment relative to its abundance has been called a keystone species, first coined by Paine (1969) and since revised extensively (Mills *et al.*, 1993; Paine, 1995). The implications of its complete extirpation or extinction cannot be accurately gauged, but it is likely to be momentous. This species is a major spring grazer, an important part of

nutrient cycling through fecal material and mucilaginous excretion for locomotion and of mineral cycling via their calcareous shells.

There are also the socio-political aspects of conservation and biodiversity. Public awareness and support for the preservation of Banff's molluscan claim-to-fame have grown over the years of Recovery Management and its mere existence provides the opportunity to learn about this unique life form and its adaptations to the harsh spring environment (Lepitzki *et al.*, 2002).

The foundation for most wilderness research and conservation projects within our borders can be summarized by the mandate of the Parks Canada Agency as per the Parks Canada Charter (2005):

"On behalf of the people of Canada, we protect and present nationally significant examples of Canada's natural and cultural heritage, and foster public understanding, appreciation and enjoyment in ways that ensure the ecological and commemorative integrity of these places for present and future generations."

With the 2002 passing of the Species at Risk Act (SARA), Parks Canada has the legal obligation to protect species that only occur within National Parks and the authority to implement necessary measures. Complementary to existing laws and agreements to provide for the legal protection of wildlife species and conservation of biological diversity, the purposes of SARA are (Lepitzki *et al.*, 2002):

"To prevent wildlife species from being extirpated or becoming extinct, to provide for the recovery of wildlife species that are extirpated, endangered or threatened as a result of

human activity and to manage species of special concern to prevent them from becoming endangered or threatened."

The process outline of species evaluation includes: Monitoring, Species assessment, Response, Recovery and Program evaluation (SARA, 2005). Execution, now and prior to SARA passing, has been undertaken for the last 32 years by a rotating-membership committee of university academics, independent specialists, Aboriginal people, government, museums or independent biologists volunteering their efforts. The establishment of the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) in 1977 provided Canadians "with a single, scientifically sound classification of wildlife species at risk of extinction" (COSEWIC, 2009). COSEWIC's mandate was originally intended for examination of vertebrates and plants, but was expanded to include molluscs, lepidopterans, lichens and mosses.

The COSEWIC process can be divided into four sequential steps:

i) Selection of wildlife species requiring assessment by the Species Specialist Subcommittees or by the Aboriginal Traditional Knowledge Subcommittee.

ii) Setting of species priority based on biological information and dividing into Groups 1 (highest priority for assessment; immediate risk of extinction or extirpation), 2 (medium) or 3 (low priority).

iii) Compilation of available species data in status reports written by qualified parties having successfully bid for the task with a detailed work plan and budget.

iv) Assessment of a species' risk of extinction or extirpation and determining its designation including: extinct, extirpated, endangered, threatened or special concern.

The related SARA process (found at sararegistry.gc.ca), follows the list above and includes an annual program evaluation, thereby completing a cyclic process leading back to monitoring, then species assessment.

There are several key criteria of which, if any single one is applicable, it warrants the designation of a species to an endangered listing. Examples of those applicable to *Physella johnsoni* include, but were not limited to:

i) 50% or greater reduction in total mature individuals over 10 years or three generations where causes are not reversible, understood or ceased.

ii) Area of occupancy is less than 500 km^2 combined with a fragmented habitat equal to or less than five locations.

iii) Quantitative population projection shows probability of extinction in the wild is 20% within 20 years or five generations up to a maximum of 100 years.

It was in April 1997 that *P. johnsoni* was designated *threatened* by COSEWIC and in 2000, a reassessment lead to its uplisting to *endangered*, defined as "a species facing imminent extirpation or extinction" (Lepitzki *et al.*, 2002).

One of the most important aspects for the Recovery Program and complete study of the Banff Spring snail is an understanding of its nutrition. Physids are known detritivores or herbivores and *P. johnsoni* has been observed to spend the majority of its time on the microbial mats, or as they are generally referred to at the Cave and Basin,

periphyton. Outlined in the holistic approach undertaken in the Recovery Program is documentation of the flora and fauna of the ecosystem and understanding their dynamics (Lepitzki *et al.*, 2002). Before the involvement of researchers from the University of Manitoba associated with this project, no microbiological surveys had been conducted at the Sulphur Mountain Springs. It has already been observed that the microbial mats serve as structural support as well as an adhesion point for the laying of eggs. It is also well-established that phototrophic microbes are primary producers of fixed carbon in aquatic environments (Overmann *et al.*, 1996; Yurkov & Bilyj, 2008). Relationships between periphyton and snails across many families have been documented throughout malacologic literature (Carlsson & Bronmark, 2006; Hann *et al.*, 2001; Kawata *et al.*, 2001; King-Lotufo *et al.*, 2002; Sheldon & Walker, 1997) and will be discussed in detail in Section 1.6. The contribution of the microbial mats to the Banff thermal spring ecology must therefore be addressed to fulfill the requirements of the Recovery Program and to understand the biology of *P. johnsoni*.

1.3 Cyanobacterial Mats and Spring Ecology

1.3.1 General Description

Thus far, "microbial mat" has been used to describe the prokaryotic consortia found at Banff Springs without defining the structural and physiological traits of this community type. Mats, or periphyton assemblages, are laminated, cohesive layered communities composed of a consortium of bacteria dominated by photoautotrophic cyanobacteria (Nisbet & Fowler, 1999; Stal *et al.*, 1985). In review literature, the distinction between

mat and biofilm appear to be subjectively qualitative in that increase of expanse and thickness lends the use of the former term over the latter, though each layer of a mat structure can be considered a biofilm in itself (Cohen & Gurevitz, 2006). In animal-associated biofilms (e.g. infections, caries), biofilms are thought of as structures only micrometers or cells thick, but in nature the thickest mats can reach several centimeters, such as those penetrating marine sediments off the coasts of Chile dominated by the massive filamentous sulfur bacterium *Thioploca* (Schulz *et al.*, 1996), or in blooms hundreds of kilometers wide as for the cyanobacterium *Trichodesmium* found in oligotrophic tropical and subtropical marine waters (Overmann & Garcia-Pichel, 2006). Microbial mats are included within definitions of biofilms sharing characteristics to distinguish from their free-floating counterparts such as population densities on the order of 10^{10} cells per ml, an extracellular polymer (EPS) matrix and a range of physical, metabolic and chemical heterogeneities exploited by dense layering of diverse species of microorganisms (De Beer & Stoodley, 2006; Nadell *et al.*, 2009). A significant fraction of inorganic and abiotic substances are also held by the EPS matrix (Stahl *et al.*, 2006). Another distinction these sessile communities have is genetic dynamism with rates of horizontal gene transfer in biofilms that are orders of magnitude higher than between cells in suspension (Ghigo, 2001) and, in *Pseudomonas*, observed changes in transcription exceeded 70% of genes in the genome compared to that of planktonic cells (Sauer *et al.*, 2002). Communal existence also provides protection from desiccation, sloughing, harmful wavelengths of light, grazing and antimicrobial agents and opportunities for metabolic symbioses, making mats ubiquitous structures in nature (Costerton *et al.*, 1995). In fact, the mat community is one of the oldest complex and

commensalistic structures on earth as evident from fossilized, layered cyanobacterial stromatolite mounds, for which a continuous geologic record covering 2.15 billion years exists (Palinska, 2008; Rasmussen *et al.*, 2008; Stahl *et al.*, 2006). In illuminated environments, cyanobacteria continue to be the dominant mat forming prokaryotes (Ferris *et al.*, 1997; Stahl *et al.*, 1985; Stal, 1995; Ward *et al.*, 1997), often with a significant presence of eukaryotic algal species (Bryanskaya *et al.*, 2006; Ferris *et al.*, 2005). The body of research on cyanobacterial mats and their distribution is vast, including those in intertidal and benthic systems (Decho, 2000; Schulz *et al.*, 1996; Stal, 1995); temperate and cold lacustrine (Hawes & Schwarz, 1999; Kisand & Noges, 1998; Taton *et al.*, 2003); hot springs (Ferris *et al.*, 1997; Hongmei *et al.*, 2005; Namsaraev *et al.*, 2003; Pierson & Parenteau, 2000), thermal springs (Chernousova *et al.*, 2008; McGregor & Rasmussen, 2008) and cool springs (Douglas & Douglas, 2001). As Winogradsky demonstrated over a century ago, typical mat community structure includes layers, often of distinct pigmentation, of metabolically related groups of organisms in order of depth: cyanobacteria and other green phototrophic organisms at the surface, purple and green sulfur bacteria, purple and green nonsulfur bacteria, chemolithic sulfur-oxidizing bacteria and a blackened bottom layer of sulfate-reducing bacteria (Palinska, 2008; Stahl *et al.*, 2006). This is not to imply a hard and fast membership in each visible layer; for example, sulfate reducing bacteria (SRB), methanogens and cyanobacteria have been found to coexist in highly oxic layers of hypersaline mat communities (Jonkers *et al.*, 2005; Minz *et al.*, 1999; Visscher & Stolz, 2005). It is rather to acknowledge that the laminated microbial communities develop vertically in response to light and to chemical and redox microgradients at the mat surface (Cohen & Gurevitz, 2006) and these

gradients create distinct microenvironments and growth structures exhibiting both spatial and temporal heterogeneity and a combination of metabolic activities that intensify environmental gradients (van Gemerden, 1993; Visscher & Stolz, 2005). A general overview of this physicochemical stratification is given below.

1.3.2 Vertical Structure and Chemical Gradients

The driving force behind the formation of phototrophic mats is light, though the optimal intensity for most cyanobacteria falls within the low range of 15-150 $\mu E \bullet m^{-2}s^{-1}$, or about 1-10% of daylight (Stal *et al.*, 1985). Pierson *et al*. (1990) wrote that the light environment is influenced by several factors including the inorganic matrix, pigments within microorganisms, extracellular pigments, dead cells, the microbial exopolymeric substances (EPS) produced and overlying water. In the same paper a survey was presented of the distribution of irradiance with depth performed by fiber optic probes moved through a mat at 100 μm increments. Results included a division of wavelength ranges 400-700 nm and 700-1000 nm finding the former decreased by 95% of its surface intensity at 1 mm depth and the latter by almost 80% at 1 mm. At about 2 mm, only 1% of incident light intensity was detected in the 400-700 nm range, while 1% of incident light in the 700-1000 nm range penetrated to almost 5 mm. Deepest penetration of these longer wavelengths was found to be 6 mm at 0.1% of surface intensity. Wavelength is inversely proportional to frequency and the energy of photons. Shorter wavelengths are higher energy and thus subject to photon scattering and shallower penetration. Interestingly, Pierson also found that wet sand allowed deeper light penetration with 0.1% of blue light reaching down to 5 mm and 0.1% infrared light penetrating close to 9 mm. As will be detailed in the following section, the light environment within a mat is

taken full advantage of by the adaptations of different photosynthetic apparatuses to absorb wavelengths of light competitively.

Cyanobacterial mats are characterized by high oxygen production during the day in the photic surface layer and by highly active sulfate reduction throughout the mat. These gradients virtually disappear at night when the entire mat turns anoxic and sulfidic. Taken from Stahl *et al*. (2006) on the distribution and conversions of oxygen, sulfur, hydrogen and carbon dioxide within a 1 cm thick phototrophic mat, O_2 is at its highest during the daytime at the surface of the mat and decreases sharply to near anoxia at 1-3 mm depth. Maximal O_2 concentrations often occur just subsurface (e.g. 0.7 mm) likely due to ideal light intensity fueling optimal oxygenic photosynthesis at that depth (Stal *et al.*, 1985). Inversely abundant to O_2 is HS^-, which is quickly oxidized to SO_4^{2-} in its presence and thus rises sharply below depths of 1-3 mm. It is in the oxic zone where oxygenic photosynthetic cyanobacteria and algae dominate the community and aerobic heterotrophic organisms, including the obligate aerobic anoxygenic phototrophs thrive (Section 1.4.4). At the bottom of this zone and below often reside chemolithic sulfur bacteria that take advantage of microaerophilic conditions to fix carbon using electrons of sulfur species released by the phototrophic sulfur bacteria at around 5 mm mat depth and HS^- from SRB below. Fermenters of photosynthesis-derived polysaccharides diffused from the surface are typically found deeper (~8 mm), converting these into organic acids and releasing H_2. The latter is oxidized by SRB and methanogens that produce CO_2 and CH_4, respectively, which effuse towards the surface. As will be expanded upon in the section on phototrophic groups, at nighttime the mat surface quickly goes anoxic within a depth of <1 mm, and thus these gradients take on a vertical diurnal pattern which can

elicit a chemotactic response from the community. It is interesting to note that analyses of complete genomes revealed that homologues of *kai* genes– those involved in "circadian regulation"– are widely distributed among both domains of prokaryotes (including cyanobacteria), indicative of a widespread ability to anticipate environmental flux (Dvornyk & Nevo, 2004). Diel shifts have been shown to be regulated by *kai* genes, members of the RecA superfamily of DNA binding proteins, involved in N-fixation, cell division and other metabolic processes (Dvornyk *et al.*, 2003; Stahl *et al.*, 2006).

Another type of gradient exists over mats in aquatic systems, known as the mass transfer or diffusion boundary layer (Riber & Wetzel, 1987). Flow hydrodynamics create an aqueous region above the mat where nutrient concentrations are lower than they are in the water column due to diminished mixing. The thickness of the boundary is positively correlated with depth and inversely correlated to flow velocity over the mat surface. Faster water currents result in a thinner mass transfer layer and increased rates of diffusion into the mat (Ghannoum & O'Toole, 2004). This leads to an interesting scenario as higher water velocity implies greater access to dissolved nutrients, but also higher shear forces on biofilms leading to the possibility of unnatural detachment or sloughing events. (Note these events may be viewed as a potential benefit as dispersal mechanisms of the mat, though the result may be that the excised portion is carried to a location where gradients are inappropriate, e.g. highly oxic and thus low sulfide waters, or water is shallow/insufficient, as may be the case with a spring stream that widens and terminates along a slope.) Conversely, lower water flow may offer two ecological obstacles for the mat community in a larger diffusion barrier and competition for dissolved nutrients from organisms in the water column above.

Now that a general picture of the structure of phototrophic biofilms has been presented, defining characteristics of each photosynthetic group creating the mat structure will be expanded upon.

1.4 Oxygenic and Anoxygenic Phototrophs in Microbial Mats

1.4.1 Cyanobacteria and Algae

Cyanobacteria are unicellular oxygenic phototrophic prokaryotes that may grow in colonies or elongated single or tight bundles of filaments. Under oxic conditions, produce chlorophyll(s) (Chl) and accessory light-harvesting pigments and typically assimilate inorganic $-HCO_3^-/CO_2$ via the Calvin-Benson cycle using light energy and perform aerobic respiration and glycogen catabolism via the oxidative pentose phosphate pathway in the dark (Stal, 1995). The photosynthetic apparatus of cyanobacteria is localized in the thylakoid membrane and comprises five functional protein complexes, four of which (photosystem (PS) II, plastoquinone-plastocyanin oxidoreductase, PSI and ATP synthase) are integral membrane proteins and one (the phycobilisome) that is peripheral. Structurally, phycobilisomes resemble the top half of an asterisk resting upon the thylakoid, above the embedded photosystems and RC, while functionally, they serve as the light-harvesting antennae of PSII (Reuter & Muller, 1993). Phycobiliprotein (open-chained tetrapyrrole phycobilins coupled to proteins) accessory pigments phycoerythrin (absorbing around 550 nm), phycocyanin (absorbing most strongly at 620-640 nm) and allophycocyanin (near 650 nm), are often found internally from the tips of the phycobilisome aggregate. Note that not all cyanobacteria contain each of these

pigments and phycoerythrin is not essential to phycobilisome formation, but the energy transfer from these complexes to Chl nears 100% efficiency. Light quanta funnel from accessory pigments to PSII, known as P680 (the chlorophyll *a* at this RC absorbs maximally at 680 nm), which has a reduction potential higher than the O_2/H_2O couple (0.82 V) and thereby is able to hydrolyze the electron donor water and release oxygen and two protons. An electron is absorbed by P680, exciting it into a moderately strong reductant that begins the electron transfer chain by reducing pheophytin (a Chl *a* molecule lacking the central Mg) before plastoquinone A, plastoquinone B, an iron sulfur protein, cytochrome *f*, cytochrome b_6 and copper protein plastocyanin, ultimately reaching the Chl dimer contained in PSI, or P700. With the energy from a photon of light, P700 (initially 0.5 V) reaches an excited state near -1.2 V to continue the second phase of electron transfer to another Chl *a* molecule, phylloquinone (a vitamin K molecule), three iron sulfur proteins (F_X, F_A and F_B), ferredoxin and finally ferredoxin NADP oxidoreductase, which reduces NADP+ to NADPH. A proton motive force is generated during the thermodynamically favorable transfer from P680 to P700 from which 1ATP is produced per molecule of water oxidized (Fork & Herbert, 1993).

The above describes "non-cyclic" electron transfer as an electron travels directly from water to NADP+, but in many species cyclic photophosphorylation involving only P700 is possible when sufficient reducing power is present. The PSI electron acceptor (the secondary Chl molecule) returns the electron destined for ferredoxin back to PSI via membrane-bound cytochromes *b* and *f* creating a membrane potential and proton motive force. This reducing power comes from electron donors other than water. Therefore this kind of photosynthesizing is anoxygenic because no oxygen is released (as found in

anoxygenic phototrophs; Section 1.4.3). A suitably reducing environment occurs under anaerobic conditions and requires sulfide or hydrogen as an electron donor, the former of the two being a known PSII-inhibitor at low (0.1-0.2 mM) concentrations (Cohen *et al.*, 1986). Extracellular sulfur crystals have been found accumulated on filaments of several species of *Oscillatoria* oxidizing sulfide while in *Microcoleus chthonoplastes* sulfide was observed to be oxidized to thiosulfate, which then served as electron donor (Cohen & Gurevitz, 2006).

These energetic pathways (known as the *light* reactions) are used to obtain sufficient ATP and reducing power to fuel the reductive pentose or Calvin cycle, for the fixation of CO_2 into an organic form suitable for biosynthesis (the *dark* reactions). This occurs in the carboxysome, an icosahedral non-membrane protein structure that contains some of the enzymes of the Calvin cycle including rate-limiting ribulose 1,5-bisphosphate carboxylase (Rubisco). The carboxysome serves as the site for a carbon concentration mechanism, a scheme involving two CO_2-uptake systems and three HCO_3^- transporters (Raven, 2003). CO_2 enters the cell by diffusion and is converted to HCO_3^- by the NDH-1 complex located in the thylakoid membrane. Transporters located in the cytoplasmic membrane carry HCO_3^- to the carboxysome where it is converted back to CO_2 by a carbonic anhydrase in close proximity to Rubisco to create 3-phosphoglycerate (3-PGA) which continues the Calvin cycle. The concentration mechanism is efficient enough that in certain environments carboxysome concentrations of inorganic C can reach 1000 times that of the external environment and CO_2 may escape the membrane to be taken up again the by the cell (Hagemann *et al.*, 2010; Ogawa & Kaplan, 1987). This C-cycling consumes a lot of light energy helping the organism combat potentially

damaging high light intensities, while the concentration of inorganic C aids in alleviating Rubisco's affinity for O_2 under highly oxic conditions. Note that this carbon flux is not omnipresent and stores of organic carbon are favored in the form of granular poly-β-hydroxybutyric acid, glycogen and EPS (Hagemann *et al.*, 2010).

The main catabolic end product of the Calvin cycle is fructose-6-phosphate, which is created in two highly energetic enzymatic steps after 3-PGA and, per molecule, requires 6 CO_2, 12 NADPH and 18 ATP to produce (Overmann & Garcia-Pichel, 2006). To help meet such demands chlorophyll production is high, with the number of molecules reaching from 200-300 per RC (compared to 30-60 bacteriochlorophyll molecules in most anoxygenic phototrophic RCs) and light energy transfer to Chl is further supplemented with carotenoid production (Glazer, 1983; Hashimoto *et al.*, 2004). Carotenoid pigments contain long hydrocarbon chains with conjugated double bonds and typically are yellow and red in color, preferentially absorbing in the blue range of the spectrum, ~440 to 520 nm (Overmann & Garcia-Pichel, 2006). Taking all accessory and Chl pigments into account, a broad range of the visible spectrum is available for light harvesting with gaps in the green-yellow and far red-infrared wavelength ranges.

The efficiency of cyanobacterial C-sequestering, storage and recycling usually satisfies metabolic demands, but during certain periods when C stores and sources in the mat near depletion and O_2 content approaches supersaturation, cyanobacteria may switch to an anabolic process called photorespiration (Eisenhut *et al.*, 2008). As mentioned above, Rubisco has affinity for O_2 as well as CO_2 and upon reacting with the former, produces 3-PGA and 2-phosphoglycolate (2-PG) (Eisenhut *et al.*, 2008). It has been known for decades that cyanobacteria release glycolate (after a dephosphorylation of 2-

PG by phosphoglycolate phosphatase), which has an important structural and trophic role in microbial mats (Stal, 1995), but actual utilization of this product by cyanobacteria was not understood until recently and seen only as a pathway necessary in plants (Hagemann *et al.*, 2010; Kaplan *et al.*, 2008). Without in-depth details of the pathway (presented in Eisenhut *et al.*, 2008), it can be noted that under highly oxic conditions, some cyanobacteria are able to convert glycolate via three paths: 1) into glyoxylate and to 3-PGA via a glycerate pathway resulting in the release of NH_4^+ and CO_2, 2) a plant-like "C2" cycle that includes glycine as a third byproduct and 3) a decarboxylation to glyoxylate, oxalate, then formate with the release of 2 CO_2 (Eisenhut *et al.*, 2008). However when these pathways are not utilized (e.g. at the *Synechococcus*-dominant mats at Mushroom Spring, Yellowstone National Park), glycolate accounted for up to 58% of total excreted photosynthates (Bateson & Ward, 1988). Other laboratory studies of algal and phytoplankton populations found up to 40% of the total fixed CO_2 was glycolate production and *in situ* studies of Yellowstone cyanobacterial mats showed 7% of photosynthetically fixed C was excreted as glycolate (Friedrich *et al.*, 1991).

The assimilation of glycolate under aerobic conditions typically requires light energy, but at nighttime, glycogen stores provide the substrate supporting the pentose phosphate pathway. Coupled with aerobic respiration, this produces reducing power in the form of NADPH, ribose-5-phosphate used for nucleic acid synthesis and erthrose-4-phosphate for aromatic amino acids. Such aerobic dark growth is not performed by all species and, when sustained, these conditions can result in cell lysis (Richardson & Castenholz, 1987b). The same study observed a diurnal migration of *Oscillatoria terebriformis* to anoxic depths as soon as light became limiting. When present,

respiration can also quickly deplete mat O_2 and many cyanobacteria must then switch to fermentative modes of growth, continuing to use glycogen. Both homo- and heterofermentation have been observed in *Oscillatoria*, the former observed in *O. limosa* producing equal amounts of lactate, ethanol and CO_2 and the latter in *O. limnetica* excreting lactate only (Cohen & Gurevitz, 2006). These and other low molecular weight compounds (acetate, formate) are arguably the most important sources of organic matter in the microbial mat offering substrates for SRB and photoheterotrophic bacteria alike (Moezelaar *et al.*, 1996; Stal, 1995).

Briefly regarding cyanobacterial species dominance in thermal springs, while there does not appear to be any environmental criterion that favors the presence of colonial over filamentous species in springs (Pentecost, 2003), many mats worldwide are dominated by filamentous *Microcoleus chthonoplastes* and *Oscillatoria* spp., as well as *Nostoc* and *Anabaena* (Stal, 1995). *M. chthonoplastes* is remarkable in having dozens of trichomes bundled in an encompassing polysaccharide sheath thus creating a protective microenvironment from desiccation and other environmental stresses, while *Oscillatoria* demonstrate high degrees of photo- and chemotaxis and a diverse metabolic scheme (Stal, 1995). The essential characteristic of *Nostoc* and *Anabaena* is nitrogen fixation. Many mats form in environments where combined nitrogen is unavailable and so these heterocyst-forming species flourish. Heterocysts are specialized non-vegetative cells that differentiate into N-fixing centers under low combined nitrogen conditions (the accumulation of 2-oxoglutarate acts as the signal in concert with a DNA-binding protease HetR) (Zhang *et al.*, 2009) and contain the key enzyme nitrogenase. Note that anaerobic conditions allow enhanced fixation of dinitrogen, as each of the components of

nitrogenase (Fe- & Mo-Fe-proteins) is inactivated by oxygen (Gallon, 1981). While many studies cite the above species as especially prevalent, the diversity of species across the range of springs is vast including *Synechococcus*, *Synechocystis*, *Spirulina*/*Arthrospira*, *Lyngbya*, *Trichodesmium*, *Phormidium* and *Calothrix* as a few of the most noted genera (Bender & Phillips, 2004; Cohen & Gurevitz, 2006; Hongmei *et al.*, 2005; Martinez-Alonso *et al.*, 2004; McGregor & Rasmussen, 2008; Pentecost, 2003; Ward *et al.*, 2006).

Photosynthetic eukaryotes, algae and diatoms, must also be addressed here briefly for their predominance in many marine, freshwater and hot spring cyanobacterial mats (Bryanskaya *et al.*, 2006; Ferris *et al.*, 2005; Martinez-Alonso *et al.*, 2004; Stahl *et al.*, 2006). Like cyanobacteria, these organisms are oxygenic phototrophs that produce chlorophylls and accessory pigments, have a noncyclic electron transport chain between two photosystems and predominantly fix carbon via the Calvin cycle. Algae also contain an inorganic carbon concentrating mechanism (Raven, 2003). A major distinction between these organisms and their prokaryotic counterparts is the compartmentalization of their photosynthetic apparatus in specialized dual-membrane intracellular organelles called chloroplasts where the thylakoids are disc-shaped and form interconnected stacks called grana. Aspects of photosynthetic eukaryotes to consider for studies in trophic significance are their cell wall structure and carbon storage compounds. Unlike cyanobacteria, algae (except euglenoids) have a cell wall composed mostly of cellulose which from a nutritional standpoint may not be as efficiently assimilated by all grazers as the cell walls of cyanobacteria. Diatom cell walls owe their rigidity to high silicon content and can feature incredibly strong holdfasts often making them physically more

resilient against grazing pressure. Typical carbon reserve materials include starches (α-1, 4-glucan, β-1, 2-glucan, etc.), sucrose, lipids, mannitol and others which typically account for ≥25% of total cell mass (Ramachandra *et al.*, 2009). Extracellular polymeric substances (next subsection) are also produced, thus offering an abundant carbon source to the next trophic level.

1.4.2 Exopolymeric Substances

Besides glycolate, fermentation products and the organisms themselves, another category of organics available to mat community constituents and higher trophic levels are exopolymeric substances (EPS). There are three broad types in cyanobacteria, the first being the endogenous polysaccharides serving as storage compounds, while the second and third are capsular or sheath polymeric substances and extracellular (colloidal) polysaccharides (Stal, 1995). The myriad organics are dominated by neutral sugars such as glucose, galactose, mannose, rhamnose and xylose and amino sugars like glucosamine, but can also include non-carbohydrate moieties (e.g. uronic and humic acids, pyruvate, succinate, nucleic acids) (Azim, 2005; Braissant *et al.*, 2009; Nicolaus *et al.*, 1999; Richert *et al.*, 2005). The production of EPS serves as a shunt for excess carbon produced during photosynthesis or, when nutrients such as nitrogen are limited while light energy is not (Wotton, 2004). Harrah (2006) describes basic bacterial exopolysaccharide production as involving a nucleoside diphosphate that modifies cytoplasmic monosaccharides to provide the basic assembly unit of the EPS. Often present are O-methylated sugars and moieties decorated with acyl groups (requiring Acetyl CoA in some systems). It was also assumed that polysaccharides synthesized at the cell membrane require a lipid such as bactoprenol (involved in peptidoglycan and

antigen O-synthesis), while phosphate groups may be added via a typical ATP-dependent phosphorylation, but more likely derived from the original sugar nucleotide. Post-polymerization may occur in the periplasm or outside the cell, but much remains unclear of the overall mechanisms (Harrah *et al.*, 2006).

As a component of mat biomass, it has been found that these substances can account for 50-90% of total organic matter with proportions of capsular and extracellular polysaccharides varying in different groups (Azim, 2005). For example, a marine study investigating two species of diatoms from *Navicula* and *Cylindrotheca* found that each secreted mostly (95%) extracellular carbohydrate (versus 5% capsular) and compared them to *Microcoleus*, which secreted 65% capsular and 35% colloidal carbohydrate (de Winder *et al.*, 1999).

Functional groups within the EPS have a high affinity for calcium and other metals, serving as mechanisms for ion exchange and entrapment (Braissant *et al.*, 2009). Mishra & Jha (2009) found amine, aromatic, halide, aliphatic alkyl-groups present in the EPS of the algae *Dunaliella salina* confirming such nutrient-sequestering potential. One study likened the adsorption of potassium nitrate and sodium bromide by a biofilm to that within an ion chromatography column and observed quite similar behavior, reinforcing that biofilms act as a trophic link between dissolved nutrients in the water column and higher levels of the ecosystem (Freeman *et al.*, 1995). Even within the same trophic level, one experiment found that removing high molecular weight dissolved materials from the water column and monitoring heterotrophic bacteria resulted in neither depletion of exogenous dissolved organic carbon nor internal carbon stores over 10 days (Freeman

& Lock, 1995). EPS was presumed the sole substrate for the maintenance of the heterotrophic population within the mat.

A wide range of bacteria produce EPS for purposes ranging from serving as a protective barrier for infectious bacteria against phagocytosis and the masking of cell-wall ligands from recognition to a means of adhesion and stabilization of biofilms (Harrah *et al.*, 2006). The result is a laminated dynamic structure that serves both the needs of the community within and offers a rich source of nutrients to other trophic levels. From here the introduction of the other key mat-associated prokaryotes follows, beginning with the anoxygenic phototrophs.

1.4.3 Purple and Green Sulfur and Nonsulfur Bacteria

Anoxygenic phototrophy is widespread across members of the *Proteobacteria* including α- (e.g. *Rhodopseudomonas, Rhodobacter, Rhodobium*) , β- (e.g. *Rhodocyclus, Rhodoferax*) and γ- (e.g. *Thiorhodococcus, Ectothiorhodospira*) subdivisions, though only the first two classes contain nonsulfur species (Imhoff, 2006). Aside from phylogenetic disparities, the general defining traits of the *sulfur* and *nonsulfur* designations are the former's preference for reduced sulfur compounds as electron donors and their storage of intracellular sulfur (or extracellular, in the case of *Ectothiorhodospira*). Purple nonsulfur bacteria mostly lack sulfur stores and generally have a lower tolerance for HS^-, concordantly exhibiting greater aerotolerance. As suggested above, these preferences dictate their positions within the mat, sulfur bacteria residing deeper to exploit anoxic zones and properties of their photosynthetic apparatuses have developed to utilize wavelengths of light able to penetrate to these depths.

Unlike the photosynthetic apparatuses of oxygenic phototrophs, the key pigment in those of anoxygenic phototrophs is bacteriochlorophyll (Bchl). Anoxygenic photosynthesis is a metabolic mode in which light is transduced into chemical energy (ATP) for growth rather than to generate both CO_2-reducing power and ATP production (Bryant & Frigaard, 2006). As in Chl, Bchl has several forms (*a*-*g*) differing in the chemical groups surrounding the magnesium tetrapyrrole structure and the phototrophs they are commonly found in. For example, purple nonsulfur bacteria generally produce Bchl *a* and *b*, which differ from each other by a substitution of Bchl *a*'s ethyl with a propylene group. Spectral properties also differ with Bchl *a* absorbing maximally *in vivo* near 870 and 800 nm and Bchl *b* at 835-850 and 1020-1040 nm. Green nonsulfur bacteria also include Bchl c_s, differing at five of the seven functional groups found in Bchl *a* and exhibiting maximal *in vivo* absorbance near 740 nm. Bchl *c, d* and *e* are found in green sulfur bacteria with major *in vivo* absorption spectral peaks at 745-755 nm, 705-740 nm and 719-726 nm, respectively. Note that the presence of oxygen downregulates formation of the internal membrane and the production of Bchl in both sulfur and nonsulfur organisms, whether facultative or strictly anaeorobic (Imhoff, 2006).

The purple/green distinction is based upon the structure of the photosynthetic apparatus, green bacteria's obligately phototrophic metabolism (following paragraphs) and the accessory pigments produced by the organisms. Carotenoid pigments do not function directly in photophosphorylation reactions, but can transmit energy to the RCs to be used in the same way as light captured by Bchl directly. Carotenoids also act as a photoprotectant able to absorb harmful radiation, scavenge singlet oxygen atoms and quench unpaired electrons in B/Chl (Frigaard *et al.*, 2004). Major series of carotenoids in

purple bacteria include spirilloxanthin, rhodopinal, spheroidene and okenone (Imhoff, 2006; van Gemerden & Mas, 1995b), while green bacteria typically produce chlorobactene and other carotene derivatives as well as several dihydroneurosporenes (Frigaard *et al.*, 2004; Imhoff, 2005a), totaling dozens of moieties absorbing wavelengths from ~420 to 560 nm. Some green sulfur strains producing Bchl *e* also produce isoreieratene, imparting a brown color on colonies (Bryant & Frigaard, 2006). It can be noted here that carotenoids are not only important from a photosynthetic perspective, but also for nutrition of upper trophic levels. Their consumption by molluscs (from oxygenic and anoxygenic phototroph sources) is beneficial for vitamin production, antioxidant action and as a carbon source (Kantha, 1989).

The purple sulfur and nonsulfur bacteria share most structural traits of the photosynthetic membrane. These intracytoplasmic membranes have various morphologies, the most common of which are lamellar, invaginations of the cytoplasmic membrane forming wide stacks, and vesicular, cytoplasmic membrane extensions that form spherical vesicles rather than elongated invaginations. Both types can be found within each phototrophic group and even within the same genus (e.g. *Rhodobacter*). Embedded within the membrane are an ATPase (residing beside the spherical protrusion in vesicular membranes) and four pigment protein complexes: the RC (also known as P870), light harvesting complex I (LHI), light harvesting complex II (LHII) and the cytochrome bc_1 complex. LHII is a donut-shaped arrangement of eight (e.g. in *Phaeospirillum molischianum*) or nine (e.g. *Rhodopseudomonas* (*Rps.*) *acidophila*) αβ-apoprotein pairs. These pairs form a ring that surrounds phospholipids from the membrane and a Bchl molecule for every protein pair, nine in total, absorbing at 800 and

820 or 850 nm depending on Bchl type (Cogdell *et al.*, 2004). Note that LHII is not essential for anoxygenic phototrophy to proceed as it is not found in all purple bacteria, for example, *Rps. viridis* (Cogdell *et al.*, 1999). The function of these LHII antennae complexes is to collect and transfer light energy to the core complex of LHI which is closely associated with the RC (Scheuring *et al.*, 2006). LHI is a horseshoe-shaped complex composed of (species-dependent) 13-16 αβ-apoprotein pairs surrounding the RC and, in some bacteria (e.g. *Rhodobacter (Rb.) sphaeroides, Rb. blasticus* and *Rb. capsulatus*), a PufX protein (Cogdell *et al.*, 2004; Scheuring *et al.*, 2006). The RC (using *Rps. palustris* as the example) contains two molecules of Bchl *a* (known as the "special pair" absorbing at ~875 nm) and is composed of two membrane-embedded central polypeptides ("L" and "M") and a third embedded protein "W" that creates the space within the LHI ring, believed to be a PufX protein analogue (Cogdell *et al.*, 2004). PufX is thought to facilitate electron transport out of the LHI-RC complex to electron carriers bacteriopheophytin and two quinone molecules (Vermeglio & Joliot, 2002). Atomic force microscopy revealed that a single vesicle in the *Rb. sphaeroides* membrane may contain up to 30 LHI-RC complexes arranged in groups of 2x3 surrounded by clusters of 100 or more LHII (Bahatyrova *et al.*, 2004). Electron transport in anoxygenic phototrophs is cyclic moving through carriers quinones A and B into a quinone pool and to cytochrome bc_1, an iron-sulfur protein and cytochrome *c* before returning to the oxidized P870, preparing it to absorb new energy. Two protons per cycle are released from the quinone pool into the periplasm to generate the motive force that drives ATP production, thus completing what is collectively called *cyclic photophosphorylation*.

Green sulfur and nonsulfur bacteria have four major distinctions in their photosynthetic apparatus: the replacement of the pheophytin-quinone with an iron sulfide protein in the RC, the presence of only one LH containing a relatively small amount of Bchl *a* (about 1% of total Bchl), a specialized protein structure in place of LHII known as the chlorosome containing all antenna Bchl *c*, *d* or *e* molecules and a unique trimer, the FMO protein, which is located between the chlorosome and RC to facilitate transduction of excited electrons (Overmann, 2001). The chlorosome is a tubular organelle lying appressed to the inside of the photosynthetic membrane and typically contains 1000-2000 molecules of Bchl *c* or *d* (Olson, 1998). As in all anoxygenic phototrophs, the concentration of Bchl molecules increases inversely to light intensity, but the sheer numerical bias in green bacteria imparts a competitive advantage in their ability to grow under incredibly low light conditions (Frigaard & Bryant, 2004). This is concordant with the strictly anaerobic, obligate phototrophic metabolism of green sulfur bacteria, preferentially utilizing reduced sulfur compounds, H_2 and ferrous iron as electron donors found deeper within the mat structure to drive autotrophy (Bryant & Frigaard, 2006).

The metabolic capacity of purple anoxygenic phototrophic bacteria is vast, including photoheterotrophy, chemoheterotrophy, fermentation, photolithoautotrophy, and chemolithoautotrophy (Imhoff, 2006; Paoli & Tabita, 1998). From a trophic standpoint though, the first three processes would be considered *secondary* production as they involve the assimilation of organic carbon from the surrounding environment including that which has been previously fixed by oxygenic phototrophs. Net organic outputs come through their capacity to use light energy or external reducing power to oxidize cytochrome *c* and drive electrons through the quinone pool against the

thermodynamic gradient to create the reducing power necessary to fix CO_2 with NADH via the Calvin cycle (van Gemerden & Mas, 1995b).

As stated above, anoxygenic phototrophs are the main driving forces of sulfur cycling within the microbial mat. Aside from photopigmentation, the capacities of sulfide oxidation, intracellular or extracellular S^0 deposition and oxidation of S^0 to sulfate are used to distinguish these major bacterial groups from each other (van Gemerden & Mas, 1995b). The cycling of sulfur by purple and green bacteria has been reviewed in depth (Canfield *et al.*, 2005; van Gemerden & Mas, 1995b) and a general summary of the most relevant reactions within these zones of the mat is presented by Van Gemerden (1993), but generally there is an energetic commensalism between sulfur groups and nonsulfur and SRB. For example, many *Chromatiaceae* and all green bacteria are able to oxidize H_2S and S^0 to $S_2O_3^{2-}$ and two green species are known to oxidize thiosulfate ($S_2O_3^{2-}$) to SO_4^{2-}, while the majority of purple nonsulfur bacteria use $S_2O_3^{2-}$, HS^- and S^0 (e.g. *Rb. adriaticus*) to form the SO_4^{2-} end product (Brune, 1989). Purple sulfur and colorless sulfur bacteria are often found in competition for electron donors between the layer of cyanobacteria and SRB taking advantage of the latter's release of sulfide. Colorless sulfur bacteria (e.g. *Thiothrix*, Section 1.5, and *Beggiatoa*) also use S^0 and HS^- for energy creating SO_4^{2-} in the process (Odintsova & Dubinina, 1993).

1.4.4 Aerobic Anoxygenic Phototrophic Bacteria

A relatively newly discovered Proteobacteria group (Harashima *et al.*, 1978), the aerobic anoxygenic bacteria (AAP) are mostly obligate aerobic, low level Bchl- and high carotenoid-producing heterotrophs that can supplement their carbon assimilatory

efficiency with light energy (Yurkov & Csotonyi, 2009). Low levels of CO_2 fixation have been found in several species, though true photoautotrophy is not possible within this group, making most of their production *secondary* (Kolber *et al.*, 2001), but their abundance– found to compose up to 11% of the total microbial community in the ocean– still proves them critical to the cycling of organic carbon in upper oceanic waters (Yurkov & Csotonyi, 2009). Like the purple bacteria, AAP have been found across a wide range of "regular" and "extreme" environments including lakes and rivers (Rathgeber *et al.*, 2005), salt springs (Csotonyi *et al.*, 2008), thermal springs (Yurkov & Beatty, 1998), near hydrothermal vents (Yurkov *et al.*, 1999) and recently soil crusts (Csotonyi *et al.*, 2010). Most AAP only use reduced carbon compounds as electron donors, though a few species (e.g. *Acidodophilium*, *Roseinatronobacter*) are able to oxidize S^0 and $S_2O_3^{2-}$, and while O_2 is the predominant electron acceptor, nitrate, nitrite, trimethylamine and ferric iron reduction has been observed (Yurkov & Csotonyi, 2009).

Some interesting traits of the AAP photosynthetic apparatus are that their amount of Bchl produced is very low (10-20%) compared to that of other anoxygenic phototrophs and while they have a large abundance of carotenoids, most of these are disengaged from the energy transduction (Yurkov & Beatty, 1998; Yurkov & Csotonyi, 2009). Most AAP only produce Bchl in the dark, though a few species (e.g. *Roseatales*, *Dinoroseobacter*), have been observed to produce 15-25% of their maximal content under illuminated conditions. AAP have a purple nonsulfur quinone-type RC and associated LHI, but variably possess LHII. The key features of the light-driven transport chain are an unusually high midpoint potential of the primary quinone acceptor Q_A, causing it to become over-reduced under anoxic conditions, and soluble cytochromes that are unable

to function anoxically, each trait halting electron flow (Rathgeber *et al.*, 2004; Yurkov & Beatty, 1998). In cyanobacterial mats, the AAP thus are found within the upper oxic layer of the mat taking advantage of the complex organics made available by oxygenic phototrophs and trapped detrital matter. While not being a source of primary production, their nutritive contribution to upper trophic layers is exemplified in the density of their carotenoids.

1.4.5 Thiothrix

Thiothrix was one of the first organisms studied by Winogradsky in his 1888 pioneering work on chemolithotrophs (Odintsova *et al.*, 1993) and a *Thiothrix*-like filamentous, colorless sulfur bacterium is also the most visibly dominant chemotroph at the Banff springs (Lepitzki, 2007). Species of *Thiothrix* are members of the γ-*Proteobacteria* and typically grow as thick white to grey-white tufts in near-neutral sulfidic waters including springs, activated sludge, sewage effluents and marine environments (Bland & Staley, 1978; Polz *et al.*, 1996). These tufts of growth are prolific and have been observed reaching proportional dominance exceeding that of cyanobacteria in illuminated environments (Chernousova *et al.*, 2008). The effective mode of this expansion is the formation of gonidia from the end of the filaments (Larkin & Strohl, 1983). A gonidium, in addition to the term's use for reproductive cells in some algae, also denotes a specialized *Thiothrix* cell covered in fimbriae capable of gliding motility, akin to the 'swarming' found in myxobacteria, and adhesion (Larkin & Strohl, 1983).

As sulfur bacteria, *Thiothrix* use HS^- and organic sulfides as energy sources and play an important role in the oxidative side of the sulfur cycle using O_2 and NO_3^- as

electron acceptors (Canfield *et al.*, 2005; Robertson & Kuenen, 2006). Four typical oxidation reactions are shown below (Canfield *et al.*, 2005):

$H_2S + 2O_2 \rightarrow H_2SO_4$ $\quad\quad$ $5H_2S + 8NO^- \rightarrow 5\ SO_4^{2-} + 2H^+ + 4H_2O$

$2H_2S + O_2 \rightarrow 2S^0 + 2\ H_2O$ $\quad\quad$ $H_2S + NO_3^- + H_2O \rightarrow SO_4^{2-} + NH_4^+$

Colorless sulfur bacteria are so named because of their lack of photopigments, but may form colored colonies at high density due to their high cytochrome content (Robertson & Kuenen, 2006). Under the microscope, multicellular filaments of *Thiothrix* appear as dense rosette formations containing innumerable refractive S^0 deposits that gradually deplete when used for oxidation under HS^--limited conditions (Odintsova & Dubinina, 1993). Other cellular features include the presence of a thin polysaccharide sheath and internal poly-β-hydroxybutyrate storage inclusions (Larkin & Shinabarger, 1983). All strains are either microaerophilic or aerobic and modes of growth are limited to chemoheterotrophy and chemoautotrophy (Polz *et al.*, 1996). Despite a narrow metabolic range, the sheer expanse that this organism reaches still offers it significant roles in carbon cycling, primary production and as a structural support and potential nutritional source for aquatic grazers.

1.4.6 Advantages of Communal Living

As already stated, the gradients of light, oxygen, sulfur species and redox couples stratify the mat community wherein different groups of phototrophic and non-phototrophic microbes cycle organic and inorganic carbon sources and electron donors and acceptors. These communities are tightly-knit with proximity being key, as the transfer efficiency of exuded metabolites decreases sharply with distance. Overmann & Schubert (2002) stated

that only 25% and 0.01% of the flux of metabolites reaches a partner cell at 2 µm and 10 µm away, respectively, compared to that of a cell at 1 µm. Exopolysaccharides stave off desiccation by binding water and, at the other end of the spectrum, provide a community-adhering matrix protective against grazing and a barrier against sloughing from high shear forces of turbulent water. Each layer of a phototrophic mat serves as a filter for particular and potentially harmful wavelengths of light, and often the very top layer is largely composed of dead cells or cyanobacteria producing the photoprotective pigment scytonemin that serves to shield organisms below (Stal, 1995).

Considering that bacteria thrive ubiquitously in the widest range of global habitats, Costerton (1995) remarked that the biofilm must be conceded "to constitute a remarkably successful life form". While pelagic forms are excellent for dissemination and are the foundational trophic level in open water aquatic systems, it is the anchored mat structure that sustains many littoral, benthic, amphibious and terrestrial organisms. The following section explores some of the relationships between these prokaryote-dominated structures and the molluscs that graze them.

1.5 Prokaryotes and Mollusc Nutrition

1.5.1 Nutrition in Physids

Because microorganisms are the only biota having the capacity to utilize the dilute carbon and energy in many aquatic habitats, the colonization and transformation of these particles by microorganisms represents an important portion of secondary production and may play an important role in food web energetics, atmospheric CO_2 exchange, and

flux of nutrients to the deep-sea ecosystem through sedimentation of colonized particles. (Stahl *et al.*, 2006).

Upper water column trophic levels are like a web of nutrient recycling between primary producers, decomposers and heterotrophic prokaryotes and metazoa (Stahl *et al.*, 2006). Fueled by nutrients (N, P and C) in the water column and inputs from primary production, heterotrophic bacteria flourish and provide sustenance to eukaryotic organotrophs while both groups produce dissolved organic matter which recycles back into the system. Exopolymeric substances also entrap detrital matter, adding to nutrient availability while often providing a physical support structure for metazoan grazing amidst water flow. What is available to molluscs then is myriad detritus and the aforementioned groups of phototrophic prokaryotes, oxygenic phototrophs, their capsular and extracellular polysaccharides, algae, diatoms, chemolithotrophic bacteria, etc. The question remains what food types are most preferable to snails. Controlled food option experiments showed that the snail *Limicolaria flammea* was capable of choosing for its dietary needs by selecting food based on somatic growth or reproductive needs, doing better on items they were free to select (Egonmwan, 1991). Previous studies of the gut contents of *Physella* relatives *P. integra* and *P. gyrina* have shown, first, little interspecies differences and that detritus was always the most common item followed by algae and exopolymer (Dillon Jr, 2000); prokaryotic biomass was not commented on. Dillon (2000) also commented that different *Physidae* have been observed to eat most anything that is available, as *Physa heterstropha* was observed to feed on most species of diatoms. A study out of a Pennsylvania woodland stream revealed 75% of gut content was algal remains even in seasons when detritus was most available. Further studies on

Physa integra confirmed the importance of oxygenic photosynthetic organisms. A biovolume study of periphyton-colonized slides demonstrated decreases in six taxa of diatoms, six taxa of algae and three each of cyanobacteria and gold-brown algae (Lowe & Hunter, 1988). Lawrence *et al.* (2002) confirmed that other *Physa* species significantly reduce algal biomass along with volumes of extracellular carbohydrate nearly 10 times the weight of oxygenic photosynthetic organisms consumed. As mentioned above, oxygenic photosynthesis-derived EPS can account for significant proportions (50-90%) of total organic matter and are often the most accessible C-source available to grazers (Azim, 2005; Hall & Meyer, 1998).

A hypothesis originally put forth by Sheldon & Walker (1997) states that microbial composition in a mat necessarily modifies the nutritional quality of plant material (e.g. detritus) for grazers. This is sound as Barker commented that herbivorous snail species face the same nutritional problems as most other herbivores: on average their bodies consist of 10% N, while their food sources only contain 4% (Barker, 2001). Dominance of heterotrophs and nitrogen-fixing cyanobacteria provide higher levels of protein and carbohydrates (a low C:N ratio) than does periphyton dominated by photosynthetic algae where polysaccharides including cellulose are also high, but protein is comparably low (high C:N ratio). An investigation of microbial degradation of leaves of New Zealand plants in water channels concluded that leaves had a C:N ratio greater than 10:1 and required microbial colonization to add nitrogen, thus "conditioning" and improving nutritional quality (Quinn *et al.*, 2000). Studies have confirmed that areas dominated by algae where snails had gone extinct exhibited higher C:N ratios than proximal sites still harboring grazers on bacterial-dominated mats of lower nutrient ratios

(C:N, 4:1) (Sheldon & Walker, 1997). Even snails that are known to be detritivorous are omnivores in that they receive energy both from detritus and from the prokaryotes that colonize it (Hall & Meyer, 1998). Interestingly there have been conflicting conclusions on the consistency of snail tissue C:N ratios. Sharfstein & Steinman (2001) studying the land snail *Pomacea paludosa* found tissue ratios consistent with food type ratios, suggesting that periphyton and detrital C:N could be used as one of the parameters in predicting resultant snail growth and reproductive rates. Qualitative measures by Fink *et al.* (2006) on the tissue ratios of four invertebrate grazers including the sea snail *Bithynia tentaculata* showed a more homeostatic control of their narrow C:N content in what was called a stoichiometric mismatch between this organism and its heterogeneous, highly variable periphyton food ratios. C and N are clearly not sole determinants of nutritive potential as most studies have found that grazers respond favorably to phosphorous enrichment, though the relationship is secondary as the response has been attributed to the increased algal biomass serving as the major food source (Sharfstein & Steinman, 2001). Regarding phosphorus, integral to the replication of genomic material, studies have found decreases in C:P ratios (as well as C:N) with mat depth explained as heterotrophs having a mean higher nutrient content than autotrophs and a strict regulation of C:N:P abundance making them more like animals than plants in terms of elemental ratio homeostasis (Makino *et al.*, 2003).

The theme arises that most molluscs are generalists, usually consuming a sequentially mixed diet, seemingly whether they are purposeful in this or not (e.g. colonized detritus). Speiser and Rowell-Rahier (1993) worked with the land snail *Arianta arustorum* and surmised that a mixed diet is preferential to keep individual

plant/cyanobacterial toxins low, to minimize antagonistic reactions of different food types lower in the gut, to complement variable nutrient contents and, as new food sources arise, grazers must constantly sample all those available to be able to choose the best foods.

1.5.2 Grazer Interactions with Periphyton

In a meta-study of 89 experiments on snail-periphyton interactions, Feminella (1995) found that grazers impacted mat biomass in 70% of studies, 81% resulted in compositional change and 60% indicated change in primary production. These results were denoted the "G → P", grazer-on-periphyton, hypothesis. Seven taxa of snails had intermediate to large effects on their periphyton substrate, while three, including the Banff snail's close relative *Physa*, had little or no effect on the mat. In the other direction, P → G, 63% of studies found that changes in abundance of mat biomass affected grazer density and 70% of the studies showed an influence on snail growth. Immediately apparent are the inconsistencies between the compiled studies' results. For example, regarding biomass accumulation, there have been disparate observations: King-Lotufo *et al.* (2002) suggested that food quality may be more of a determinant in *P. virgata* since periphyton biomass accumulation did not consistently influence grazing rate estimates, while Hill (1992) found the opposite trend in the land snail *Elimia clavaeformis*, stating that grazers can compensate for low quality simply by increasing grazing rates.

What was consistent in most studies, including those on *P. virgata*, was that high snail densities depressed grazing and thus growth rates (Feminella, 1995; King-Lotufo *et*

al., 2002). At higher numbers molluscs expended more energy on interference behaviors like avoidance or shaking their shells after contact with another individual, and less time grazing than they did at intermediate densities. At such populations, mollusc grazers may affect periphyton biomass, rates of production and general heterogeneity of the mat (Kawata *et al.*, 2001) in negative ways, but studies have also shown that grazer inputs can be useful sources of nutrients for heterotrophic bacteria within mats. Liess & Haglund (2007) reasoned that snail slime trails can be a source of phosphorous and excrement a source of nitrogen, and in their study on *Theodoxus fluviatilis* found a significant increase of the inorganic N in the water column. They concluded that direct grazing effects are *less* important than the effect of nutrient inputs, when nutrients are limited and grazer density is low. Haglund & Hillebrand (2005) similarly found an increase in bacterial biomass due to nutrient recycling when grazers were present. Grazing may also have an effect by consumption of dead algae on top layers, thus increasing the light availability to living cells below (Steinman, 1996).

A study of the relationship that might exist between *P. johnsoni* and the microbial mats at Banff Springs will take a multi-faceted approach, which our lab sought to initiate with this project. This introduction ends with the goals of this pioneering work.

1.6 Project Goals

For over a decade several researchers have collaborated towards developing an understanding of the population dynamics of *P. johnsoni* and the chemical and hydrological factors governing the springs. It was in 2003 that Dr. Vladimir Yurkov was

invited by Parks Canada aquatic specialist Charlie Pacas to elucidate microbiological aspects of this unique environment, with a focus upon Bchl-containing and oxygenic photosynthetic microorganisms.

In accordance with the Recovery Plan objectives and the pure research interests of our laboratory on the subject of microbial photosynthesis, the following goals comprised this project:

1) Seasonal comparative enumerations and cataloguing of the major phototrophic organisms forming the microbial mat. Sampling throughout the year was done with the goals of representation of each of the four seasons and replication where achievable, as would be necessary to map out an annual pattern of microbial community membership and dominance. Fieldtrips from Winnipeg, Manitoba to Banff, Alberta were essential to minimize storage time of spring mat samples taken for enumeration in culture media developed for the selection and enrichment of the three groups of anoxygenic phototrophs and for growth of oxygenic photosynthetic cyanobacteria and microscopic eukaryotes. As previously mentioned, heterotrophic organisms ultimately rely on the organic matter produced by green plants and other oxygenic phototrophic species to meet their food requirements, except for the minor amounts associated with chemoautotrophic production facilitated by inorganic carbon and energy sources (Fahey & Knapp, 2007). Ascertaining the types of organisms present within the springs contributing to this production and tracking their patterns of distribution throughout the spring for comparison to *P. johnsoni* population data was interesting from the microbial ecological viewpoint and essential to the understanding the snail's enumeration flux. Observation of changes in cyanobacterial mat expanse, the changes in absorption spectrum profiles at

different times and quantification of Chls *a* and *b* and Bchl *a* content with the mat were included in the analysis.

2) The quantitative analysis of seasonal photosynthetic activity of the microbial mat via incorporation of ^{14}C-labelled sodium bicarbonate. Relative rates of production in phototrophic mats have been found comparable to those of tropical rainforests (Jorgensen, 2001; Krumbein *et al.*, 2003). As expressed by Fahey and Knapp (2007), primary productivity is the rate at which energy is stored in the organic matter of photosynthetic organisms per unit area, often expressed in units of mass because of the ease in conversion to energy for plant tissues. As stated above this is the fixation of atmospheric or aquatic CO_2 driven by photosynthesis and by the chemical energy from inorganic molecules via the Calvin cycle in oxygenic phototrophs or, by low redox electron donors or reverse electron flow in anoxygenic photoautotrophs. *Gross* primary production can be thought of as the rate of energy fixed or total organic material created by plants, algae, diatoms, photosynthetic prokaryotes and lithotrophs, while *net primary production* describes the organics remaining after use in maintaining tissue and fulfilling the respiratory needs of these producers. We quantify net primary production because our insight into the dynamics of organic carbon available to higher trophic levels depends fundamentally upon the knowledge of patterns and controls of this kind of production.

3) Isolation and taxonomical description of novel, potentially endemic strains exhibiting unusual morphological or spectral characteristics. As microbiologists, we are perpetually interested in new phenotypic characters and deducing why they appear in a particular niche. Biodiversity is of equal interest to Parks Canada to incorporate into

their species catalogues and to increase the notoriety of the Banff Springs area as unique and deserving of conservation efforts.

2 Materials and Methods

2.1 Collection of Samples

Samples were first collected in August 2003 from 13 sites including both the Upper Middle Springs (UMS; N51° 09' 50", W115° 34' 53") and the Cave and Basin (C&B) Historic Tourist Site (N51° 10' 05", W115° 35' 24"), the two locations separated by approximately 100 m elevation and 2.7 km drive along Sulphur Mountain (~1 km straight-line). Representative 1 cm^2 mat specimens were cut from each site, measured for thickness and placed into 1.5 ml Eppendorf tubes containing 1 ml of spring water and put on ice to be used for enumeration. A second set of samples was obtained and stored on ice in 50 ml plastic tubes (Falcon) for community spectrophotometry and microscopy. All sites, with the exception of a discontinued location "Site 2" (a submerged mat not colonized by *P. johnsoni*), were sampled in subsequent fieldtrips during May 2004; February, May and October 2005; March 2006 and May, August and November 2007. Sampling also occurred in December 2005 specifically for community spectral analysis.

2.2 Isolation, Enumeration and Enrichment of Bacteria

Isolation and enumeration procedures were done on media designed for purple and green sulfur bacteria (PSB), purple and green non-sulfur bacteria (PNSB) and aerobic anoxygenic phototrophs (AAP). Samples were resuspended in spring water and

decimally diluted to 10^{-8}. Aliquots of 0.1 ml were dispensed into anaerobic agar (0.6%) deeps containing purple and green non-sulfur bacterial (PNS) medium or purple and green sulfur bacterial (PS) medium and spread onto plates of rich organic (RO) medium. Each medium was adjusted to pH 6.8-7.0. PNS contained (g/l): $MgCl_2$, 0.5; KH_2PO_4, 0.3; NH_4Cl, 0.3; $CaCl_2 \bullet 2\ H_2O$, 0.1; Na-acetate $\bullet$ 3 H_2O, 1.0; malate, 0.3; yeast extract, 0.2 and (ml/l): trace elements, 2.0 (Drews *et al.*, 1983); vitamin solution, 2.0 (Yurkov *et al.*, 1999); L-cysteine (0.3 mM), 5.0; L-methionine (0.3 mM), 5.0 and spring water, 50. PS contained (g/l): $MgCl_2$, 0.5; KH_2PO_4, 0.3; NH_4Cl, 0.3; $CaCl_2 \bullet 2\ H_2O$, 0.1; Na-acetate, 0.1; yeast extract, 0.05 and the following solutions (ml/l): trace elements, 2.0 and vitamins, 2.0 (each as above); $NaHCO_3$ (10%), 20; $Na_2S \bullet 9\ H_2O$ (10%), 3.5; and spring water, 50. RO agar (2%) plates contained (g/l): $MgSO_4 \bullet 7\ H_20$, 1.0; KH_2PO_4, 0.3; NH_4Cl, 0.3; $CaCl_2 \bullet 2\ H_2O$, 0.1; Na-acetate, 1.0; yeast extract, 1.0; Bactopeptone, 0.5 and casamino acids, 0.5; and (ml/l) each of trace elements and vitamin solutions, 2.0 (as above). Strains BF1, BF16 and BF14 (Section 3.2) were isolated in PNS agar deeps while strains BF9 (Section 3.3) and BF8 (Section 3.4) were obtained on RO agar plates.

Agar deeps were incubated in the light at 27^{o}C for 14 days before enumeration and RO plates were grown in the dark at 28^{o}C for 10 days before first enumeration and 14 days for the second enumeration. All colonies were categorized by morphotype and color and screened for Bchl as described below.

For culturing of cyanobacteria and oligotrophic aerobic phototrophs, 0.1 ml aliquots of sample dilutions were spread-plated onto a variation of BG11 agar (1.5%) plates (Waterbury, 1992) adjusted to pH 6.8-7.0. Modified BG11 contained (g/l): $MgSO_4$

• 7 H_2O, 0.075; $CaCl_2$ • 2 H_2O, 0.036; $NaNO_3$, 0.15; KH_2PO_4, 0.03; Na_2CO_3, 0.02; Na_2-EDTA • 2 H_2O, 0.001; ferric ammonium citrate, 0.006; citrate, 0.006; and (ml/l): trace elements and vitamin solution, 1.0 each (as above) and spring water, 35.

Liquid and agar (2%) pyruvate mineral salts (PMS) medium was used for anaerobic enrichment. PMS was adjusted to pH 6.8-7.0 and contained (g/l): Na_2-EDTA • 2 H_2O, 0.01; $MgSO_4$ • 7 H_2O, 0.2; $CaCl_2$ • 2 H_2O, 0.075; NH_4Cl, 1.0; K_2HPO_4, 0.9; KH_2PO_4, 0.6; Na-pyruvate, 2.2; yeast extract, 0.1 and (ml/l): trace elements and vitamin solutions, 2.0 (as above); L-cysteine (0.1 M), 5.0; L-methionine (0.1 M), 5.0 and spring water, 50. Inoculated Balch tubes were incubated at 25 °C under incandescent light. Plates were grown in anaerobic jars in a 27 °C illuminated incubator.

A modified version of PE medium (Hanada *et al.*, 1995) was used in aerobic plating. PE contained (g/l): Na-glutamate, 0.5; Na-succinate, 0.5; yeast extract, 0.5; Casamino acids, 0.5; $Na_2S_2O_3$, 0.5; KH_2PO_4, 0.38; K_2HPO_4, 0.39 and (ml/l): vitamin solution, 1.0 and the above referenced Hanada *et al.* basal salt solution without NaCl, 5.0.

2.3 Spectral Analysis

Spectral analysis of pigmented colonies and community samples was performed as previously described by Yurkov and van Gemerden (1993a). Absorbance spectra were recorded between 350 and 1100 nm at room temperature with a U2010 spectrophotometer (Hitachi, Tokyo, Japan). Estimation of chlorophylls *a*, *b* and *c* was calculated as previously reported (SCOR-Unesco, 1966).

2.4 Light Microscopy

Phase-contrast microscopy was done at 1000x magnification with an Axioskop 2 Light Microscope and digital camera (Zeiss). Micrographs of the bacterial community were processed using Northern Eclipse software.

2.5 Photosynthetic Activity and Carbonates Measurement

Community photosynthetic and chemosynthetic carbon fixation was measured by 24-hour on-site incubation of 1 cm^2 mat with C^{14}-labelled Na-bicarbonate (Steemann Nielsen, 1952) in May and October, 2005; March, 2006; and May and November, 2007.

Carbonate and bicarbonate levels were determined by titration method (Franson, 1998) in May and October, 2005; March, 2006 and August 2007. Temperature at each site was obtained using an alcohol thermometer (Kessler) and measurements of pH were made using a portable pH meter (Fisher).

2.6 Physiological and Biochemical Tests

For photoheterotrophic experiments, strains BF1, BF14, BF16 and BF9 were grown on a succinate minimal salt (SMS) medium, which had the same composition as PMS medium above except for the replacement of Na-pyruvate with an equal mass of Na-succinate. A solution of the first six PMS components served as a basal salts medium for taxonomic tests including: single carbon source utilization (concentrations as in Imhoff & Caumette, 2004), carbohydrate fermentation (sugars added at 0.1%), autotrophy (with 0.1% $NaHCO_3$), vitamin requirement, starch (0.2%) and gelatin (12%) hydrolysis (Tindall *et al.*, 2007). For the nitrate reduction test, NH_4Cl was replaced by 0.1% KNO_3 and yeast extract was omitted. Results were read as described in Tindall *et al*. (2007). Motility

was tested in Motility Agar (Fisher Scientific Canada) and observed by hanging drop method (Tindall *et al.*, 2007). Tests for hydrolysis of Tweens 20 and 60 (Fisher Scientific Canada) at 1.0% (v/v) were done on SMS plates with $CaCl_2$ increased to 0.1 g/l (Tindall *et al.*, 2007). The growth optima of pH and temperature, salinity and sulfide tolerances were checked in pH-adjusted liquid medium and optical density was read with a Klett meter or by absorption values at 660 nm spectrophotometrically. Oxidase Spot-test reagent was obtained from Becton Dickinson and Company. Nitrogen source preference and test for autotrophy with select electron donors were executed as recommended by Imhoff and Caumette (2004). Test for fermentation of sugars was done as previously described in Rathgeber *et al.* (Rathgeber *et al.*, 2005). Antibiotic discs were purchased from BBL (of Becton Dickinson and Company) and testing was carried out as in Yurkov & van Gemerden (1993b).

Characterization of strain BF8 was carried out in the same manner as above using RO medium or the inorganic components of RO as the basal salts medium for carbon substrate utilization and all other applicable taxonomic tests.

2.7 Genomic Analysis

DNA G+C content was determined by HPLC (Simadzu apparatus) with DNA hydrolysis and nucleotide dephosphorylation carried out as described in Mesbah *et al*. (1989) under conditions adapted from Tamaoka and Komagata (1984). Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and direct, almost-complete sequencing of the purified PCR products (e.g. 1454 nucleotides for strain BF9) were

carried out as described by Rainey *et al*. (1996). Phylogenetic trees were inferred within the ARB package (Ludwig *et al.*, 2004) by using evolutionary distance (neighbor-joining DNAPARS algorithm with Felsenstein correction) and the PHYLIP program for maximum parsimony (Felsenstein, 1989).

3 Results

3.1 Abundance and Diversity of the Phototrophic Microbial Mat Communities of Sulphur Mountain Banff Springs and their Significance to the Endangered Snail, *Physella johnsoni.*

3.1.1 Study Site Description

Three groups of springs on Sulphur Mountain presently support populations of *P. johnsoni*: Kidney Springs, the C&B National Historic Site and the UMS; the latter two chosen for this project. Sites (Fig. 2) were chosen for their proximity to snail aggregations (Sites 3, 5, 6 and 10-12), for microbiological interest (Sites 1, 2, 4, 7, 8 and 9) or as a comparative control (Site 13, no snails). Site 1 was located within the cave of the C&B. The Cave contains an 8-10 m wide and 0.5-1.5 m deep pool with a 0.5 m wide outflow stream. Within the turbulent outflow, a flocculent cream-white bacterial mat grew attached to the surface of the underlying rocks. Dim illumination came from sunlight through a 1 m grating, 6 m above the pool surface and an incandescent lamp approximately 4 m from the mat. Site 3 was designated within the deep green microbial mat on the surface of the Basin spring outflow pool. The expanse of the growth was determined largely by the floating detritus supporting it. A Site 2 was chosen from a mat

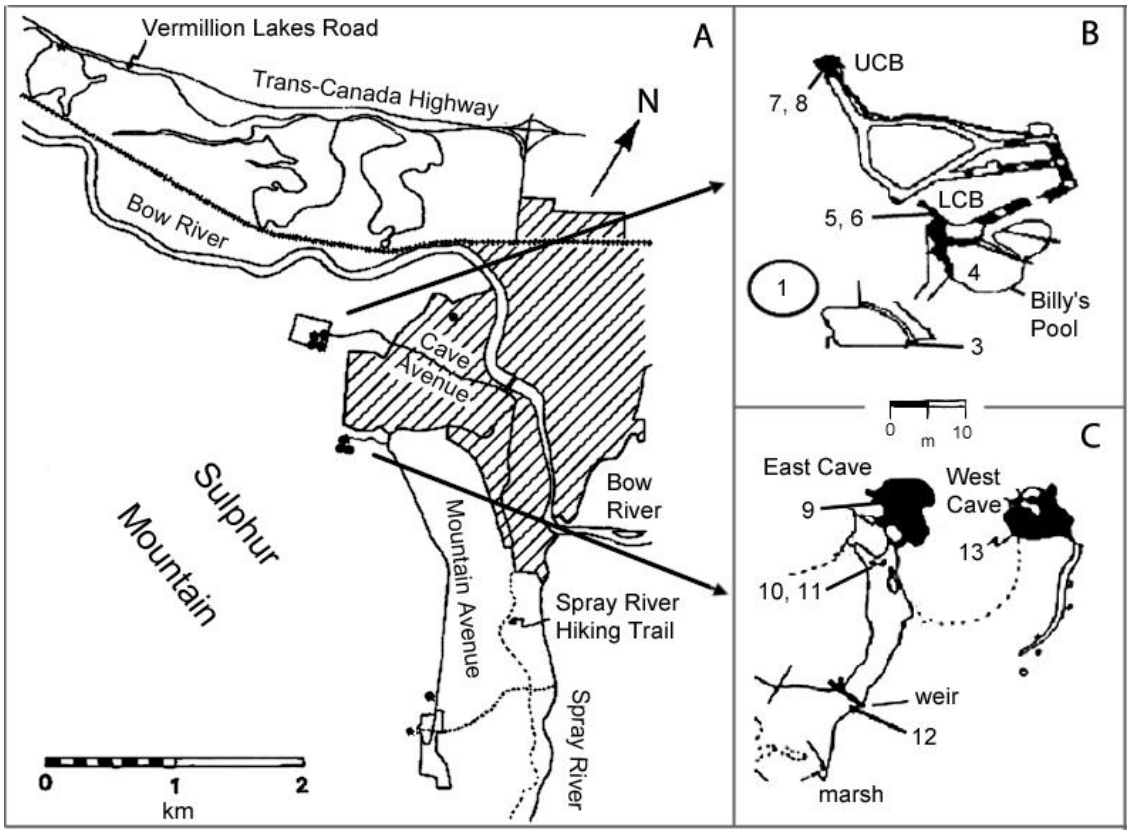

Fig. 2. Map of the Banff Sulphur Mountain thermal springs area (A) including the Cave and Basin Historic Tourist Site (B) and the Upper Middle springs restricted area (C). Sampling locations Sites are indicated by numbers 1-13. Black dots in (A) indicate individual springs; lined area is Town of Banff; UCB, Upper Cave and Basin spring source; LCB, Lower Cave and Basin spring source.

~1 m below the Basin pool surface, but was discontinued after August 2003 due to obstacles of winter sampling and because snails were never observed at such depths. Site 4 was located in an open 10-12 m diameter, 0.1-0.5 m deep pool (known as Billy's Pool), dense with aquatic plants and, according to Lepitzki and Pacas (2007), the small "mosquito fish" *Gambusia affinis*. Samples were taken from the dominant orange formation just subsurface of the pond. Site 5 was located within stream of the Lower Spring of the C&B that feeds Billy's Pool. This location typically had a lush, white

midstream microbial development attached to rocks and branches source-side of a boardwalk that crosses over this stream. The deep green component of the microbial mat, adjacent the white layer of Site 5, was distinguished as Site 6. At the Upper C&B spring, Site 7 was sampled from the thin pink-purple mat (Fig. 3A) and Site 8 was the green subsurface portion of the microbial mat next to Site 7.

The remaining sites were located at the UMS. Site 9 was the thin dark olive green mat within the UMS cave approximately 20 cm underwater near the spring source. Sunlight was limited due to cave structure and midday light intensity was measured ~1.3 $\mu E\ m^{-2}\ s^{-1}$. Average water temperature was 35.2 °C, highest of all sampling locations (see Table 1). Site 10 was approximately 5 m from the opening of the source cave and sampling was from the white flocculent growth on the surface of rocks within spring stream. Site 11 was the deep green portion of the microbial mat (Fig. 3B) located near Site 10. Site 12 developed approximately 10 m further from Site 11 on the downstream side of a wooden weir. Samples were taken from the white and pale green mat present within the turbulent outflow caused by the weir. Site 13, located 10 m laterally from the UMS stream, grew at the mouth of the West Cave (WC) (Lepitzki, 2002), was uninhabited by *P. johnsoni* and was chosen for comparison of bacterial populations. The samples were collected from a thin purple and green mat 1-4 cm subsurface along the edge of the cooler rainwater-fed pool (average temperature 25.2 °C).

3.1.2 Macro- and Microscopic Analysis of Natural Samples

Table 1 summarizes the data of several seasonal observations. Micrographs of Site 1 consistently revealed *Thiothrix*-like filaments as the sole dominant component. Large,

amorphous refractile intracellular particles were common, indicating the oxidation of sulfide and the accumulation of elemental sulfur typical of *Thiothrix* (Fig. 3J). Ovoid cells were visible in significant proportions only in May and August 2007, corresponding to its thickest development of 7 mm from 4 mm observed in October. The mat developing the greatest and most variable thickness was Site 3 on the Basin pool surface, observed at 4 mm in March and over 20 mm in August (Table 1). Giant straight green filaments hundreds of micrometers in length were noted only at this site, qualitatively predominant in May and August 2007. These unbranching filaments (Fig. 3C) were 20-26 µm diameter and had sheaths with near-rectangular cells separated by deep constrictions, distinguishing them from most species of *Oscillatoria* and *Lyngbya* in size and frequency of cell division (Cohen & Gurevitz, 2006). In colder sampling months of February, March and October, a fluctuating majority between *Phormidium* and/or *Microcoleus*, *Spirulina* (Fig. 3F) and *Oscillatoria*-like (Fig. 3E) species was revealed. The orange subsurface mat of Site 4 was only available for analysis five of nine samplings. The orange color was contributed by the aerobic growth of a C*hloroflexus*-like organism (Fig. 3K) observed only in August 2003 and, in future samplings, coccoid aerobic phototrophic relatives of *Erythromicrobium* and *Porphyrobacter* (Fig. 3P). Strains of these two genera of AAP were subsequently cultured in large proportions at multiple sites at both C&B and UMS systems (see Section 3.1.4). Unicellular *Aphanothece*- and *Dermocarpa*-like (Fig. 3I) and pennate diatoms similar to *Amphora* (Fig. 3H) were also observed in spring and summer months. The Site 4 mat was thickest in May 2005 measured at 4 mm, but was subsequently lost before the October sampling and virtually undetectable in all seasons except August 2007 at <1 mm.

Table 1. Summary of seasonal observations showing collection dates, organisms or morphotypes dominant in each sample as viewed by a light microscope, thickness of the mat sample, Site temperature, pH, carbonate and bicarbonate levels, sulfide (HS^-) and dissolved oxygen (DO) levels; maximum peaks of whole-community spectral analysis and mat development expressed as a percentage of the maximum development observed in August 2003. HS^-, DO from Lepitzki 2007. Blanks indicate not determined (nd).

Site	Date	Dominant Organism(s)	Mat (mm)	Temp[1] °C	pH	-HCO3 (mg/l)	-CO3 (mg/l)	HS^- (mg/l)	Dissolved oxygen	*In vivo* Community Spectral Peaks[3] (nm)	Relative % Development[2]
	3-Aug-2003	*Thiothrix*	5	32	7.1					n/a	100
	11-May-2004	*Thiothrix*	nd	31						n/a	20
	17-Feb-2005	*Thiothrix*	5	30	7.1					n/a	90
	19-May-2005	*Thiothrix*	4	30	7.0					n/a	20
1	30-Oct-2005	*Thiothrix*	4	30	7.0					n/a	30
	21-Mar-2006	*Thiothrix*	5	30.5	6.9					n/a	30
	6-May-2007	*Thiothrix,* cocci	4 - 5	31	7.0					n/a	30
	13-Aug-2007	*Thiothrix,* bent rods	6 - 7	30	6.8					n/a	70
	9-Nov-2007	*Thiothrix*	5	30	6.9					n/a	60
	3-Aug-2003	giant filaments	10	32.5	7.1			4.3	0.16	446, 483, **622**, 677	100
	11-May-2004	*Phormidium, Spirulina*	nd	33.5	7.1			4.89	0.84	(418), 443, (626), 683, **733**, 802, 852	50
	17-Feb-2005	*Oscillatoria*	6	31.5	7.2			3.85	0.95	(393, 420), 439, (489), **621**, 677	20
	19-May-2005	*Phormidium, Spirulina*	10	33	7.1	128	9	4.09	0.73	(427), 450, 620, **679**, 722, 803, 853	50
3	30-Oct-2005	*Spirulina, Oscillatoria*	5	31.5	7.1	134	9.6	3.74	0.87	441, (491, 561), 618, **679**	50
	21-Mar-2006	*Spirulina*	4	32	7.1			4.23	1.66	438, 623, **679**	60
	6-May-2007	giant filaments, *Phormidium, Spirulina*	6 - 7	32.5	7.0			3.36	1.43	442, 621, 680, **727**, 788, 856	vandalism, 60
	13-Aug-2007	giant filaments , *Oscillatoria, Spirulina*	7 - 25	33	7.1	129	19.8	3.59	0.9	439, 621, **678**, 735, 850	60
	9-Nov-2007	*Spirulina, Phormidium, Oscillatoria*	13 - 16	32	7.1	134	12	4.14	2.16	448, 490, 569, 622, **678**	50
	3-Aug-2003	*Chloroflexus*	nd	28	7.0					(418), 439, 485, 620, **675**, (798), 869	100
	11-May-2004	*Chloroflexus*, algae/*Synechocystis*	nd	23.5	nd					435, 481, (610), **673**	50
	17-Feb-2005	*Chloroflexus*	2	25	7.1					421, 438, 489, 547, 566, 624, **678**	10
	19-May-2005	*Oscillatoria*	4	30.5	7.0					438, 482, **616**, 675	50
4	30-Oct-2005	nd	nd	nd	nd					nd	<1
	21-Mar-2006	nd	nd	nd	nd					nd	<1
	6-May-2007	nd	nd	nd	nd					nd	<1
	13-Aug-2007	algal cells, *Chloroflexus*-like, diatoms	1	25	6.9					438, 480, 620, **674**	10
	9-Nov-2007	nd	<1	nd	nd					nd	<5
	3-Aug-2003	*Thiothrix*	nd	34	7.6			1.04		(412, 616, 674)	100
	11-May-2004	*Thiothrix*	nd	33	7.6			1.04		n/a	50
	17-Feb-2005	*Thiothrix*	4	33	7.6			0.82		n/a	10
	19-May-2005	*Thiothrix*	3	33	7.6	121	9.6	0.5		n/a	50
5	30-Oct-2005	nd	nd	31	7.6			0.62		nd	nd
	21-Mar-2006	nd	nd	33	7.6			0.67		nd	nd
	6-May-2007	*Thiothrix,* coccoid-ovoid cells	7	33	7.4			0.74		n/a	40
	13-Aug-2007	*Thiothrix,* algal cells, *Beggiatoa*	6 - 10	32	7.5	124	8.7	0.59		(406, 620, 674)	50
	9-Nov-2007	*Thiothrix*	4 - 5	32	7.6	115	12.6	0.72		n/a	70
	3-Aug-2003	*Oscillatoria*	10	34	7.6			1.04		419, 438, 490, **625**, 675	100
	11-May-2004	*Oscillatoria*	nd	33	7.6			1.04		410, (436), 589, **677**, (802, 852)	50
	17-Feb-2005	*Phormidium*	4	33	7.6			0.82		438, **627**, 679	10
	19-May-2005	*Oscillatoria*	3	33	7.6	121	9.6	0.5		445, 621, **683**	50
6	30-Oct-2005	nd	nd	31	7.6			0.62		nd	nd
	21-Mar-2006	nd	nd	33	7.6			0.67		nd	nd
	6-May-2007	*Oscillatoria*, coccoid-ovoid cells	5	33	7.4			0.74		440, (492), 630, **679**, (852)	50
	13-Aug-2007	*Oscillatoria,* algal cells, *Phormidium*	3	33	7.5	124	8.7	0.59		413, **674**, 722, 824	60
	9-Nov-2007	*Oscillatoria,* algal cells, *Beggiatoa*	3 - 4	33	7.6	115	12.6	0.72		416, 438, **613**, 676, (719), 800, 852	90
	3-Aug-2003	*Chromatium, Phormidium*	1	33	7.1			3.16	0.21	417, 436, **674**, 749, 825	100
	11-May-2004	*Spirulina, Phormidium*	nd	31.5	7.1			3	0.54	423, 669, 745, (798), 824	90
	17-Feb-2005	*Chromatium, Phormidium*	1	33	7.2			2.45	1.7	(595), 670, 746, 798, 857	10
	19-May-2005	*Chromatium, Spirulina*	3	32.5	7.1	134	10.2	2.84	0.68	442, (675), **743**, (798), 824	90
7	30-Oct-2005	*Chromatium, Phormidium*	7	32	7.2	136	8.4	2.24	0.67	417, (435), **672**, 741, (825, 850)	20
	21-Mar-2006	*Chromatium*	1	32	7.2			2.16	1.41	514, 730, **825**	80
	6-May-2007	nd	4	32	7.0			3.25	0.65	nd	20
	13-Aug-2007	*Chromatium,* some *Phormidium*	2 - 3	33	7.1	139	8.7	3.04	1.25	415, 673, 724, **826**	90
	9-Nov-2007	*Chromatium,* some *Phormidium*	4 - 5	34	7.0	134	7.2	3.33	0.51	442, 675, **723**, 801, 851	90
	3-Aug-2003	*Spirulina*	4	33	7.1			3.16	0.21	445, 494, 678, 737, 804, **825**, 886	100
	11-May-2004	*Spirulina, Phormidium*	nd	31.5	7.1			3	0.54	(424), 441, 679, **727**, (802), **824**, 858	50
	17-Feb-2005	*Phormidium*	5	34	7.2			2.45	1.7	441, 681, **737**, 824, 849	10
	19-May-2005	*Phormidium*	3	32.5	7.1	134	10.2	2.84	0.68	446, 620, 685, **741**, 823, (854)	50
8	30-Oct-2005	*Spirulina*	6	32	7.2	136	8.4	2.24	0.67	414, **670**, 722, (851)	80
	21-Mar-2006	*Spirulina, Phormidium*	3	30	7.2			2.16	1.41	438, 620, **678**, (825)	70
	6-May-2007	*Spirulina, Phormidium, Beggiatoa*	3	32	7.0			3.25	0.65	445, **674**, (860)	50
	13-Aug-2007	*Phormidium, Spirulina,* algal cells	1 - 2	33	7.1	139	8.7	3.04	1.25	420, 617, **676**, 726, (804), 824, (843)	70
	9-Nov-2007	*Phormidium, Spirulina, Oscillatoria*	3 - 4	33	7.0	134	7.2	3.33	0.51	409, 672, 726, (750), **828**, 847	110

Site	Date	Dominant Organism(s)	Mat (mm)	Temp[1] °C	pH	-HCO3 (mg/l)	-CO3 (mg/l)	HS^- (mg/l)	Dissolved oxygen	*In vivo* Community Spectral Peaks[3] (nm)	Relative % Development[2]
	3-Aug-2003	*Phormidium*	nd	35	nd					756	100
	11-May-2004	*Phormidium*	nd	34.5						749	60
	17-Feb-2005	*green cocci, Phormidium*	2	36	7.0					451, **752**	50
	19-May-2005	*green cocci, Phormidium*	3	35.5	7.1					457, (630, 668), **755**	60
9	30-Oct-2005	*Phormidium, Beggiatoa*	10	34	7.0					746	20
	21-Mar-2006	*Phormidium,* bacilli	12	36	7.0					673, **752**	60
	6-May-2007	*Phormidium, Beggiatoa*	2	36	6.9					461, **758**	70
	13-Aug-2007	*Phormidium, Beggiatoa,* ovoid cells	2 - 3	36	7.0					431, 455, **753**, (867)	90
	9-Nov-2007	*Phormidium, Beggiatoa,* ovoid cells	6	36	6.9					451, **752**	100
	3-Aug-2003	*Thiothrix*	nd	35	7.1			3.74	0.4	418, 437, 620, **678**, 749, (854)	100
	11-May-2004	*Thiothrix*	nd	35	7.0			3.78	1.03	n/a	50
	17-Feb-2005	*Thiothrix*	4	34	7.1			3.27	1.14	(671, 749)	40
	19-May-2005	*Thiothrix, Spirulina*	6	35	7.1	123	9	3.91	0.72	439, 619, **679**, 743	50
10	30-Oct-2005	*Thiothrix*	4	34	7.1	132	8.4	2.94	0.77	n/a	25
	21-Mar-2006	*Thiothrix*	4	34	7.1			3.25	1.13	742	50
	6-May-2007	*Thiothrix*	3	34.5	6.9			4.14	1.11	n/a	50
	13-Aug-2007	*Thiothrix, Phormidium*	4 - 5	35	7.1	132	9.1	2.76	2.18	437, 624, 679, 747, **851**	90
	9-Nov-2007	*Thiothrix, Phormidium*	4 - 5	35	7.0	129	8.4	3.66	0.72	413, 453, **748**, 849	90
	3-Aug-2003	*Spirulina*	nd	35	7.1			3.74	0.4	420, 440, 490, 622, **679**	100
	11-May-2004	*Spirulina*	nd	35	7.0			3.78	1.03	394, 419, 438, 491, (591), 623, **679**	50
	17-Feb-2005	*Spirulina*	4	33	7.1			3.27	1.14	424, 439, 622, **679**, 749	60
	19-May-2005	*Spirulina*	5	34.5	7.1	123	9	3.91	0.72	(395, 419), 438, (587), 624, **678**	50
11	30-Oct-2005	*Spirulina*	3	32	7.1	132	8.4	2.94	0.77	440, 614, **679**, 746, 853	10
	21-Mar-2006	*Phormidium, Spirulina*	4	34	7.1			3.25	1.13	438, 623, **678**, 750	40
	6-May-2007	*Spirulina, Oscillatoria*	6	33.5	6.9			4.14	1.11	440, (492), 630, **679**, (852)	50
	13-Aug-2007	*Spirulina*	6 - 7	34	7.1	132	9.1	2.76	2.18	439, (488), 624, **678**	70
	9-Nov-2007	*Spirulina*	3	34	7.0	129	8.4	3.66	0.72	439, 625, **678**, 751, 851	80
	3-Aug-2003	*Thiothrix, Oscillatoria*	5	35	7.8			3.74	4.93	(419), 442, 484, 623, **678**, 749, 849	100
	11-May-2004	*Thiothrix, Phormidium*	nd	34	7.7			3.78	4.67	419, 437, 589, **623**, 678	50
	17-Feb-2005	*Thiothrix, Oscillatoria*	2	33	7.7			3.27	4.05	415, 435, **623**, 678	40
	19-May-2005	*Thiothrix, Phormidium*	4	34	7.7	110	13.8	3.91	4.0	439, (490, 586), 625, **678**	50
12	30-Oct-2005	*Thiothrix, Oscillatoria*	4	33	7.7	134	7.8	2.94	4.05	(440, 626, 679)	60
	21-Mar-2006	*Thiothrix, Phormidium*	6	33	7.8			3.25	4.54	438, **623**, 678, 749	70
	6-May-2007	*Thiothrix, Oscillatoria*	5	34	7.5			4.14	4.45	438, **627**, 679	50
	13-Aug-2007	*Thiothrix, Oscillatoria*	7 - 11	35	7.7	122	10.8	2.76	4.53	418, 437, 621, **678**, 747	90
	9-Nov-2007	*Oscillatoria, Thiothrix, Phormidium*	6	34	7.7	115	12	3.66	nd	415, **621**, 677, 749, (853)	90
	3-Aug-2003	*Spirulina, Oscillatoria*	5	28	6.9					414, 437, 621, **678**, 850	100
	11-May-2004	*Phormidium, Spirulina*	nd	23.5						421, 438, 494, 624, **679**	90
	17-Feb-2005	*Lyngba*	7	22	6.9					439, 567, 625, **680**, 718, 824, 851	50
	19-May-2005	*Phormidium, Oscillatoria*	9	27	7.0					439, 624, **680**, 757, 824, (849)	90
13	30-Oct-2005	*Oscillatoria, Spirulina*	2	26	7.0					(415), 438, **618**, 678, 754	15
	21-Mar-2006	*Spirulina, Oscillatoria*	7	21	6.9					437, **623**, 678, 750	20
	6-May-2007	*Phormidium, Beggiatoa, Spirulina*	1	27	7.0					443, 628, (685), 726, **832, 858**	80
	13-Aug-2007	*Phormidium, Spirulina, Oscillatoria*	2	26	6.9					420, (626), 675, **753**, 835	90
	9-Nov-2007	*Phormidium, Oscillatoria, Spirulina*	1	26	6.8					439, (562, 625), 679, 723, **826**, 851	80

[1] Water temperature at the surface of the mat taken with an alcohol thermometer to the nearest half degree

[2] Relative percent development: the seasonally observed areal expanse of the bacterial mat expressed as a percentage of the maximum coverage at that site witnessed in August 2003.

[3] Maximum absorption peaks of sonicated whole-community mat samples expressed in nm. Wavelengths in bold indicate highest non-cytochrome peaks and those in brackets reflect weak, less discernible absorption values.

Site 5 May and August samples had single-celled oxygenic phototrophs of *Synechocystis* morphology and motile ovoid cells as minor members during its maximal development of approximately 1 cm thickness. Minimal thickness was measured in February and May 2005 before the mat was found absent in October 2005 until summer 2006. Site 6 of the Lower C&B spring (3-4 mm thickness) had a significant presence of *Phormidium*-

/Microcoleus-like species, *Spirulina* and *Dermocarpa*-like cells with consistent dominance by *Oscillatoria*-like filaments in all seasons. As at Site 5, the mat was lost in 2005 after a summer and autumn of unusually high precipitation. June 2005 rainfall values were more than four times greater than the monthly averages recorded over the last 51 years, while September experienced over twice the average precipitation (Lepitzki, 2007).

The thin purple-pink mat of Site 7 was composed predominantly of large ovoid cells resembling purple sulfur *Chromatium* (Fig. 3T) and rounded *Thiocapsa* (Fig. 3U) each containing multiple, highly-refractive sulfur inclusions. Amongst these cells, filaments of *Phormidium* were part of the cellular matrix in all seasons. Adjacent to Site 7 was the green mat of Site 8, dominated alternately by filamentous species of *Spirulina* and *Phormidium* and attaining maximum thickness in autumn and winter months, October and February (Table 1).

Within the UMS cave, the thin olive green mat (Site 9) was consistently populated by *Phormidium* sp. filaments. A co-dominant morphotype was similar to *Chlorobium limicola*, as a green coccoid chain-forming bacterium that was observed throughout the year (Fig. 3V). *Oscillatoria* and *Spirulina* were visible in February, March, May and October. Small white tufts were scattered atop the mat, revealed by microscopy to be *Beggiatoa*-like filaments with multiple refractive inclusions. The *Thiothrix*-like mat at Site 10 showed no single-celled morphotypes and little fluctuation in thickness was measured (3-6 mm). In summer and autumn 2007, a layer of *Phormidium* and

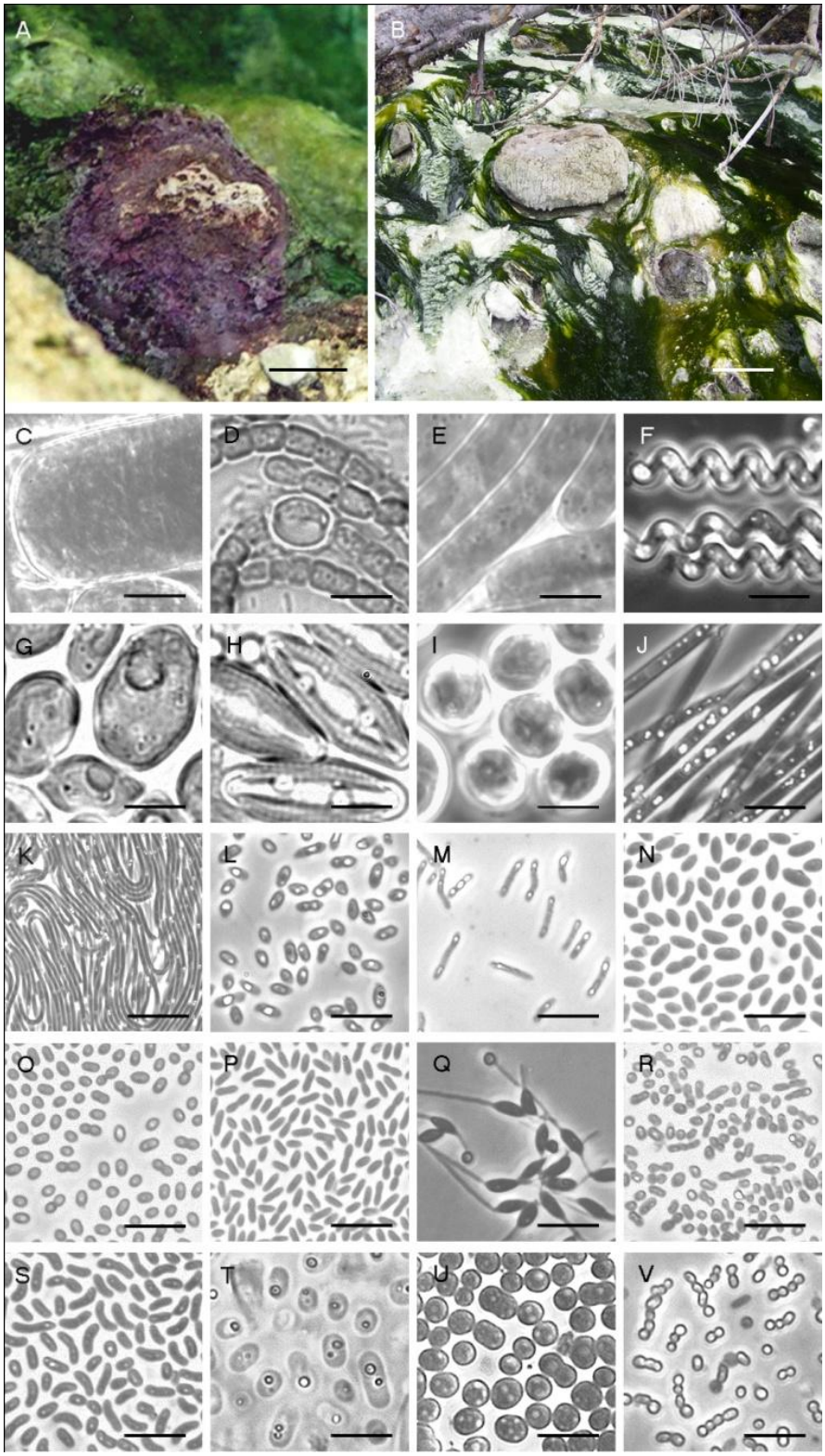

Fig. 3. Site photos and phase-contrast micrographs of morphological diversity observed at the Banff springs. Listed genera are morphologically similar or were determined by 16S rRNA sequencing with

percent relatedness indicated in brackets. A) purple mat at Site 8, UCB; B) *Thiothrix*- and *Spirulina*-dominated development within the UMS stream, C) small portion of unknown *Oscillatoria*-like filaments, D) *Anabaena*-like chains, E) *Oscillatoria*-like filaments; F) *Spirulina*-like filaments; G) *Chlorococcum*-like oxygenic phototroph; H) *Amphora*-like diatoms, I) *Dermocarpa*-like oxygenic phototroph, J) *Thiothrix*-like filaments; K) *Chloroflexus*-like filaments, L) *Rhodobacter capsulatus* (98.0%), M) *Rhodopseudomonas palustris* (99.0%), N) *R. capsulatus* (formerly *Rhodopseudomonas capsulata*; 98.0%), O) *Roseomonas lactus* (99.9%); P) *Porphyrobacter tepidarius* (99.0%), Q) *Rhodomicrobium vannielii* (99.6%), R) pink AAP strain BF60, S) *Paracraurococcus ruber* (93.7%), T) *Chromatium*-like cells, U) *Thiocapsa*-like cells, V) *Chlorobium*-like cocci. Bar A, 5 cm; Bar B, 20 cm; Bars C-V, 10 μm.

Microcoleus filaments was developed beneath complete top layer of *Thiothrix*, creating an interesting structural arrangement. Site 11 was dominated by *Spirulina* in all seasons with a presence of *Phormidium* sp. filaments in February, March, May and October. Site 12 had the most turbulent water flow and supported a *Thiothrix*-dominated mat that varied in thickness from 3-4 mm in all 2005 samplings to over 1 cm in August 2007. During warmer months the mat was interwoven with light green filaments of *Oscillatoria* (Fig. 3E) while a more visible presence of *Phormidium* and *Microcoleus* filaments, *Spirulina* (Fig. 3F), *Anabaena* (Fig. 3D) and single-celled species resembling *Aphanothece* occurred in October, November and February. Control Site 13 lacked *Thiothrix*, though morphotypes from all other sites were observed: *Beggiatoa* as a minor component in all seasons and cyanobacterial representatives included *Phormidium* (a major morphotype in all seasons), *Anabaena* (minor in November), *Oscillatoria* (a significant component in all seasons), *Spirulina* (minor in all seasons) and sheathed *Lyngbya* (a major community member in February) (Table 1).

3.1.3 Seasonal Community Spectrum Analysis

Spectral analysis was used to ascertain pigments that were most abundant in different seasons, reflective of the dominance of different phototrophic organisms within the mat community and availability for *P. johnsoni* grazing. Consistent with predictions, Chl *a* was the main photosynthetic pigment (absorption peaks in the 661-664 nm range) throughout all seasons for Sites 3, 6, 8, 11, 12 and 13. Concentrations of Chl *a*, *b* and *c* were calculated in October, December, March, May, August and November for wet sample mass (Table 2) thus results were intended for comparison to each other and *P. johnsoni* counts. As expected, samples 3 and 11 from the thickest and greenest photosynthetic mats had the highest Chl *a* concentrations (maximums of 3043 and 3785 µg Chl *a*/g wet mass at Sites 3 and 13, respectively, in October), though Site 13 was consistently Chl-rich exhibiting values from 212 to 2231 µg Chl *a*/g wet mass. *Thiothrix*-dominant samples contained the least; typically <50 µg Chl *a*/g wet mass at Sites 1 and 5

Table 2. Chl *a*, *b* and *c* values in µg Chl/g microbial mat for Banff Springs sampling sites at six collections between October 2005 and November 2007. Proportions were calculated from wet samples and are therefore an underestimate of actual Chl contained per mass of mat. ns indicates not sampled.

Site	µg Chl/g of mat																	
	Oct-05			Dec-05			Mar-06			May-07			Aug-07			Nov-07		
	Chl a	Chl b	Chl c	Chl a	Chl b	Chl c	Chl a	Chl b	Chl c	Chl a	Chl b	Chl c	Chl a	Chl b	Chl c	Chl a	Chl b	Chl c
1	4	7	21	47	0	0	1	0	1	0	0	0	0	0	1	0	0	0
3	3034	466	0	1129	1316	1606	393	102	141	823	772	0	148	110	69	905	183	7
4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	79	29	42	ns	ns	ns
5	ns	ns	ns	ns	ns	ns	ns	ns	ns	35	0	0	31	6	5	5	3	10
6	ns	ns	ns	ns	ns	ns	ns	ns	ns	104	29	1	173	207	24	160	79	0
7	1166	747	0	700	755	0	96	42	0	ns	ns	ns	165	150	0	98	221	0
8	1168	567	0	416	475	234	194	67	46	834	680	0	70	37	0	210	199	0
9	558	0	5	553	0	78	89	1	31	631	0	51	98	0	0	65	0	0
10	100	2	0	22	1	2	15	0	3	15	9	1	126	24	8	22	0	0
11	3785	351	118	1600	648	694	317	32	57	626	42	40	200	14	1	646	201	111
12	116	5	3	186	0	0	53	4	8	143	9	1	42	10	13	17	27	117
13	2231	167	0	1996	364	391	363	21	22	212	452	0	475	204	0	786	1059	0

and <130 µg Chl *a*/g wet mass at Site 10 in all experiments. Such comparatively low Chl *a* values at Sites 1, 5 and 10 were a simple reinforcement of the typical stratification of mats that render oxygenic phototrophs separate from the *Thiothrix*-dominant portions of the mat expanse. As revealed by enumeration of pigmented strains (following section), cohabitation within the *Thiothrix* development was generally reserved for PNSB.

Generally higher Chl *a* concentrations were obtained in cooler months of October, November and December. This was consistent with the indirect relationship between light intensity/availability and production of photosynthetic pigments, which thereby enables greater harvest of the diminished luminosity (Geider, 1987; Post *et al.*, 1985). A similar phenomenon exists in pure culture anoxygenic phototrophs, where PNSB and PSB induce greater Bchl *a* production to ameliorate photon absorption in low light (Drews & Gorlecki, 1995), though in comparing community Bchl concentrations to Chl values (Fig. 4), disparate patterns of fluctuation between the two pigments could be seen at different sites. Fig. 4A and 4B display somewhat opposing trends in the seasonal measures of Chl and Bchl at sites 3 and 12, respectively, while in contrast Sites 10, 11, 8 and 13 (Fig. 4C-F) exhibited a more correlated change. For spectra, samples 10 and 11 were taken from an area that *P. johnsoni* did not access and thus, like Site 8 and 13, could experience no grazer impact, while Sites 3 and 12 were the most populated with the snail. Although limited sampling trials, the possibility exists that a dense population of *P. johnsoni* could have influenced the fluctuations of pigments by influencing the fluctuation of bacterial populations. If *P. johnsoni* were to graze intensely enough, the implications could have been to impose physical damage to oxygenic phototrophs thus affecting mat Chl content. Snails may also have served as an input of organics through

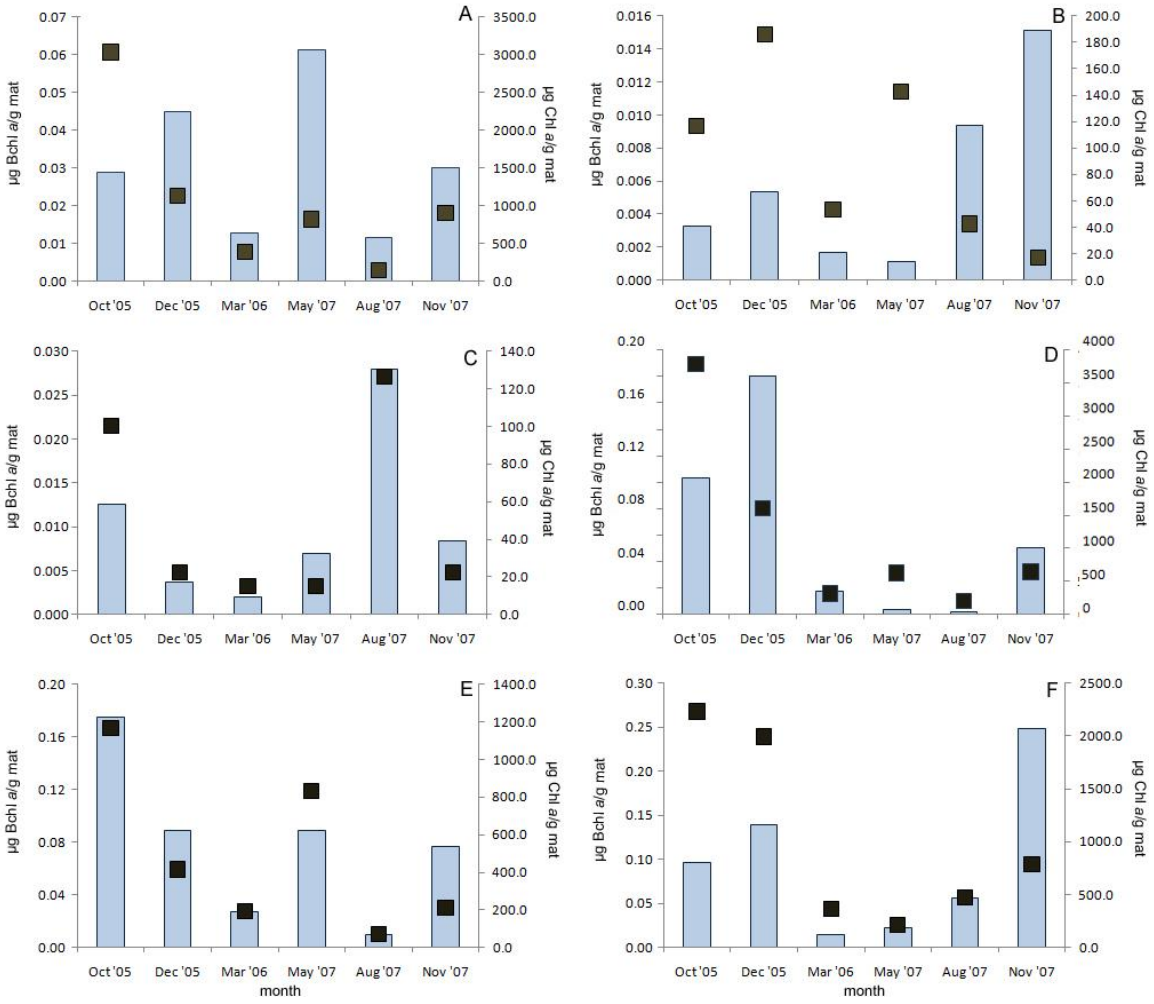

Fig. 4. Comparison of mat Chl *a* (dark squares) and Bchl *a* (bars) content (in µg of pigment per gram wet mass of mat) obtained in each sampling season at A) Site 3, B) Site 12, C) Site 10, D) Site 11, E) Site 8 and F) Site 13.

fecal matter and slime trail excreta and, in concert with grazing upon and removing light-shielding surface cyanobacteria, may have offered increased nutrients and illumination to spur photoheterotrophic proliferation of PNSB deeper in the mat. Low detection of Bchl at the time of the largest mat thickness and development (e.g. May or August) could also suggest an increased reliance on purely heterotrophic metabolism when covered by a thicker layer of oxygenic phototrophs, especially for PNSB at light-limited depths of

greater than 9 mm (Pierson *et al.*, 1990). It must be acknowledged that no definitive conclusion can be made without an increased number of sample trials.

Table 3. Bchl *a* values expressed as µg Bchl/g microbial mat for Banff Springs sampling sites in select seasons. Regarding Chl, data were obtained from wet samples and are an underestimate of actual Bchl contained per mass of mat. ns indicates not sampled.

	µg Bchl/g of mat					
Site	Oct-05	Dec-05	Mar-06	May-07	Aug-07	Nov-07
1	0.030	0.002	0.001	0.001	0.001	0.005
3	0.029	0.045	0.013	0.061	0.012	0.030
4	ns	ns	ns	ns	0.007	ns
5	ns	ns	ns	0.007	0.006	0.002
6	ns	ns	ns	0.006	0.029	0.014
7	0.192	0.217	0.059	ns	0.087	0.016
8	0.175	0.089	0.027	0.089	0.010	0.077
9	0.103	0.159	0.011	0.076	0.051	0.047
10	0.013	0.004	0.002	0.007	0.028	0.008
11	0.104	0.181	0.018	0.004	0.002	0.051
12	0.003	0.005	0.002	0.001	0.009	0.015
13	0.097	0.139	0.015	0.022	0.056	0.249

3.1.4 Enumeration of Anoxygenic Phototrophs

Regarding aerobic anoxygenic phototrophs (AAP), the highest counts were obtained August 2003 at the outflow basin of Site 3: $2.3x10^5$ CFU/cm^2 of mat, equivalent to 4% of all pigmented bacteria or 2.3% of total heterotrophic bacteria enumerated on RO plates. The 2003 value was over 200 times greater than August 2007 values (equivalent to 1.1% of pigmented isolates and 0.4% of total heterotrophs enumerated), over 17 times more CFU/cm^2 than obtained in May 2005 (23% of pigmented colonies, 1% of total heterotrophs) and over 23 times more numerous than in November 2007 (4.4% of

pigmented isolates, 3.1% of all heterotrophs). As seen in Table 1, increase in mat thickness might suggest increase in habitat for AAP from season to season, though a typical cyanobacterial mat will become anoxic at night below depths of around 3 mm (Stal *et al.*, 1985) thereby inhibiting the flourish of obligate aerobes like AAP. While the May 2005 (1.32×10^4 colony-forming units, or CFU/cm^2) and November (1.00×10^4 CFU/cm^2) figures (Table 4) are only ~9 times that of August 2007 (1.43×10^3 CFU/cm^2), the 20 times larger Aug. 2003 value of 2.33×10^5 CFU/cm^2 might be explained by comparing *P. johnsoni* populations enumerated each August. At 2104 snails, the August 2003 count was more than double the August 2007 census (Lepitzki unpublished) therefore, as described above for PNSB, the potential for simultaneous input of organics (e.g. fecal matter) and the removal of surface cyanobacteria (by grazing) by *P. johnsoni* existed to spur heterotrophic growth of AAP. In other months, AAP declined to anywhere from 100 CFU/cm^2 in March 2006 to non-detection (<1 CFU/cm^2) in February and October 2005 and May 2007.

The second highest AAP enumeration occurred at the submerged orange mat of Site 4, possibly reflecting the higher oxygenation of this shallow, plant-laden pool. The August 2007 sampling yielded 3.0×10^4 CFU of orange ovoid AAP per cm^2 of mat, equal to 35.4% of all aerobic anoxygenic phototrophic isolates, and 9.6×10^5 CFU/cm^2 (18.9% of total RO plate isolates) in May 2005. The Upper Cave and Basin (UCB) cyanobacterial mat (Site 8) yielded the third highest density of AAP also in May 2005 at 9.3×10^4 CFU/cm^2 or 9.5% of all RO medium heterotrophic isolates, followed by 1.9×10^4 CFU/cm^2 enumerated in May 2007.

The Site 1 Cave only yielded AAP in October 2005 (2.2 x 10^3 CFU/cm^2) and in May 2007 (238 CFU/cm^2). With abnormally high precipitation in summer 2005, it is possible that excessive runoff combined with any sloughing of Lower C&B mats led to an abundance of bacterial transfer beyond typically observed spring streams. These waters may have served as a vector for transfer of phototrophic bacteria from the higher elevated sampling locations to Site 1 through the Cave's overhead opening. A low maximum of 8.2 x 10^2 CFU/cm^2 was observed at Site 10 in May 2005 and overall the seasonal mean for AAP at the three *Thiothrix* mat sites (1, 5 and 10) was a mere 90 CFU/cm^2.

PNSB were also found in peak abundance at Site 3 in August 2003 at 4.8x10^5 CFU/cm^2. Subsequent enumerations showed variance from as low as 90 CFU/ cm^2 in February 2005 to 6.6 x 10^3 and 8.1 x 10^3 CFU/cm^2 in May and August 2007, respectively. Unexpectedly the next highest density of PNSB was discovered within the turbulent UMS stream (Site 12) at 3.15 x 10^5 CFU/cm^2 in February followed by 2.0 x 10^5 CFU/cm^2 in October 2005. The *Spirulina*-dominated mat of Site 11 showed low PNSB counts (<200 CFU/cm^2) in all seasons except the two autumn samplings: 1.7 x 10^3 and 8.0 x 10^4 CFU/cm^2 in October 2005 and November 2007, respectively. Lowest average PNSB counts arose from the dimly lit *Thiothrix*-dominated Site 1 at an average <1 x 10^2 CFU/cm^2 with the exception of 1.1 x 10^4 CFU/cm^2 detected in August 2007.

Table 4. Number and proportion of Bchl *a*-containing isolates of total bacteria enumerated on selective media in all sampling seasons.

Site	Medium	Bchl-containing and total bacteria enumerated at Sulphur Mountain Spring sites																					
		February '05		% of	March '06		% of	May 2007		% of	August 2003		% of	August 2007		% of	October '05		% of	November '07		% of
		Bchl	total	total	Bchl	total	total	Bchl	total	total	Bchl	total	total	Bchl	total	total	Bchl	total	total	Bchl	total	total
1	RO	<1	$2.04x10^4$	0	<1	4851	0	238	61950	0.38	<1	$2.00x10^8$	0	0	$2.19x10^5$	0	2226	$1.35x10^4$	16	<1	$4.75x10^4$	0
	PNS	40	$1.70x10^4$	0.23	44	1674	2.6	78	$1.51x10^5$	0.05	200	$9.80x10^6$	0	$1.08x10^4$	$6.08x10^4$	17.70	12	3112	0.39	63	$6.96x10^4$	0.09
	PS	1	7001	0.01	11	7111	0.15	1001	$5.55x10^4$	1.8	0	$4.80x10^5$	0	70	130	53.85	510	1790	28	2	1252	0.16
3	RO	<1	$1.10x10^4$	0	100	$4.51x10^4$	0.22	<1	$1.80x10^6$	0	$2.33x10^5$	$9.97x10^6$	2.33	1438	$3.26x10^5$	0.44	<1	$1.71x10^5$	0	$1.00x10^4$	$3.20x10^5$	3.1
	PNS	90	9990	0.90	144	8344	1.7	6605	$5.39x10^4$	12	$4.78x10^5$	$2.18x10^6$	21.88	8100	$2.12x10^4$	38.21	1170	$2.61x10^4$	4.5	757	9757	7.8
	PS	200	9236	2.2	$1.10x10^4$	$1.61x10^5$	6.9	<1	10	0	<1	$2.00x10^7$	0.00	6600	$1.06x10^4$	62.26	50	$2.81x10^4$	0.18	600	$1.36x10^4$	4.4
4	RO	$3.48x10^4$	$4.64x10^5$	7.5	nd	nd	n/a	nd	nd	n/a	664.1	$2.41x10^4$	3	$2.95x10^4$	$8.33x10^4$	35	nd	nd	n/a	nd	nd	n/a
	PNS	$2.62x10^4$	$1.32x10^4$	20	nd	nd	n/a	nd	nd	n/a	300	$7.22x10^5$	0.04	515	$2.85x10^4$	1.81	nd	nd	n/a	nd	nd	n/a
	PS	$7.40x10^5$	$1.85x10^6$	40	nd	nd	n/a	nd	nd	n/a	<1	4140	n/a	nd	nd	n/a	nd	nd	n/a	nd	nd	n/a
5	RO	<1	$5.20x10^6$	0	nd	nd	n/a	nd	nd	n/a	<1	$1.61x10^6$	0	<1	$2.55x10^6$	0	nd	nd	n/a	<1	$1.56x10^6$	0
	PNS	410	$2.01x10^5$	0.20	nd	nd	n/a	nd	nd	n/a	<1	7600	0	1807	$1.61x10^7$	0.01	nd	nd	n/a	2400	6150	39
	PS	650	$9.30x10^5$	0.07	nd	nd	n/a	nd	nd	n/a	<1	760	0	5400	$1.70x10^5$	3.2	nd	nd	n/a	210	$4.42x10^4$	0.48
6	RO	<1	$3.87x10^6$	0	nd	nd	n/a	<1	$1.65x10^6$	0	34	$6.10x10^4$	0	<1	$2.00x10^6$	0	nd	nd	n/a	<1	$7.65x10^6$	0
	PNS	4202	$9.64x10^5$	0.44	nd	nd	n/a	3330	$3.43x10^5$	0.97	<1	$1.38x10^9$	0	3450	$1.08x10^6$	0	nd	nd	n/a	2900	$2.10x10^6$	0.14
	PS	4600	$8.25x10^5$	0.56	nd	nd	n/a	nd	nd	n/a	$2.00x10^6$	$2.82x10^8$	1	$6.00x10^4$	$9.60x10^5$	6	nd	nd	n/a	1420	$3.41x10^5$	0.42
7	RO	<1	$1.10x10^7$	0	99	$4.34x10^4$	0.23	nd	nd	n/a	<1	$2.60x10^5$	0	1000	$4.40x10^5$	0.23	<1	$1.12x10^5$	0	201	$9.62x10^4$	0.21
	PNS	nd	$1.50x10^4$	n/a	2602	8377	31	nd	nd	n/a	<1	$1.02x10^7$	0	$1.65x10^4$	$6.75x10^4$	24.44	$1.15x10^4$	$9.65x10^4$	12	<1	$7.35x10^4$	0
	PS	$4.95x10^4$	$5.53x10^4$	90	15700	$4.16x10^5$	3.8	nd	nd	n/a	2200	10800	20	$9.50x10^4$	$1.37x10^6$	7	4019	$1.02x10^6$	0.40	680	2680	25
8	RO	<1	$3.92x10^6$	0	<1	$2.64x10^4$	0	$1.86x10^4$	$9.56x10^5$	1.95	1000	8240	12	<1	$3.36x10^5$	0	798	$3.51x10^5$	0.23	<1	$3.96x10^5$	0
	PNS	nd	$8.00x10^4$	n/a	768	1248	62	$5.17x10^4$	$4.05x10^6$	1.27	400	5060	8	$1.80x10^4$	$1.48x10^5$	12	$3.08x10^4$	$1.41x10^5$	22	8500	8500	100
	PS	9960	$1.53x10^4$	65	1330	$1.91x10^5$	0.70	nd	nd	n/a	200	$1.16x10^4$	2	$5.86x10^4$	$2.74x10^5$	21	$5.19x10^5$	1.02x106	51	$2.08x10^4$	$4.08x10^4$	51
9	RO	<1	$4.95x10^5$	0	<1	$1.02x10^5$	0	nd	nd	n/a	<1	1600	0	<1	$1.04x10^7$	0	<1	$2.80x10^4$	0	<1	$4.00x10^4$	0
	PNS	11	733	1.5	<1	1730	0	$1.00x10^4$	$1.10x10^4$	91	<1	380	0.0	201	3801	5.3	2730	$2.47x10^4$	11	3020	3020	100
	PS	14	2635	0.53	$2.20x10^4$	$1.52x10^5$	14	300	$1.00x10^6$	0.03	<1	320	0.0	28	28	100.0	$1.10x10^5$	$1.81x10^5$	61	61	61	100
10	RO	<1	1518	0	9	$2.62x10^4$	0.03	280	4555	6.15	147	$3.05x10^4$	0.48	nd	nd	n/a	<1	$1.91x10^4$	0	61	$1.77x10^4$	0.35
	PNS	11	1728	0.64	150	1710	8.8	509	7509	6.8	8700	$4.16x10^4$	20.9	4200	$1.26x10^4$	33.4	3890	5190	75	400	900	44
	PS	$1.4x10^4$	$2.13x10^4$	66	1030	$1.54x10^4$	6.7	1750	$1.01x10^5$	1.7	$5.80x10^5$	$7.87x10^5$	73.70	600	1000	60.00	2700	$1.34x10^4$	20	680	980	69
11	RO	4	963	0.42	1	$5.35x10^4$	0	301	5400	5.57	40	3950	1.0	$1.35x10^4$	$2.95x10^5$	4.6	1	5761	0	<1	$3.55x10^6$	0
	PNS	130	460	28	7	$6.50x10^5$	0	190	1890	10	20	$1.02x10^5$	0	$2.11x10^4$	$7.78x10^4$	27	1721	7621	23	$8.01x10^4$	$1.22x10^5$	66
	PS	5	451	1.1	55	945	5.8	120	8120	1.5	<1	8940	0	6000	$1.20x10^4$	50	500	1700	29	$1.11x10^5$	$1.22x10^5$	91
12	RO	21	$2.74x10^7$	0	<1	$4.70x10^5$	0	<1	$4.72x10^5$	0	$1.77x10^4$	$5.75x10^5$	3.08	<1	$2.04x10^6$	0	<1	$3.41x10^5$	0	201	$2.39x10^7$	0
	PNS	$3.15x10^5$	$7.51x10^7$	0.42	170	$3.80x10^5$	0.04	8300	$3.11x10^6$	0.27	$7.40x10^4$	$7.75x10^5$	9.55	1450	$3.51x10^5$	0.41	$2.00x10^5$	$4.00x10^5$	50	$1.51x10^4$	$2.22x10^6$	0.68
	PS	$3.30x10^4$	$1.90x10^6$	1.7	725	$6.21x10^5$	0.12	$1.24x10^4$	$5.61x10^6$	0.22	<1	$7.60x10^4$	0.0	3280	$4.22x10^4$	7.8	5796	$3.80x10^4$	15	580	$1.50x10^5$	0.39
13	RO	<1	$4.63x10^4$	0	<1	2966	0	<1	$1.40x10^4$	0	92	$7.01x10^4$	0.13	401	$2.00x10^5$	0.20	<1	3100	0	200	5520	3.6
	PNS	$3.24x10^4$	$5.24x10^4$	62	7500	$1.38x10^4$	54	700	700	100	$1.40x10^4$	$5.20x10^4$	27	8145	9495	86	3440	7440	46	620	3620	17
	PS	$2.6x10^4$	$6.81x10^4$	38	$3.85x10^4$	$1.20x10^5$	32	3400	7400	46	$1.00x10^4$	$2.60x10^4$	38.5	nd	nd	n/a	1820	1953	93	80	80	100

nd – not determined; n/a – not applicable

Rhodomicrobium-like isolates exhibiting the characteristic ovoid to elongate-ovoid cells and production of exospores were one of the most frequently enumerated PNSB from all Sites excluding 1, 5 and 10. Site 7 yielded consistently highest counts from 1.3 x 10^3 to over 1.0 x 10^4 CFU/cm^2 (February and October, respectively). Sites 12 and 13 proved the next most abundant for this morphotype in fall to early spring ranging from 1.2 x 10^2 to 1.0 x 10^3 CFU/cm^2 (Site 12 for October and February) and 7.0 x 10^2 to 1.0 x 10^4 CFU/cm^2 (Site 13 in October and May 2007), respectively.

Numerous single-celled chlorophyll *a*-containing algae were isolated in PNS and PS anaerobic deeps abundantly from all sites reaching 1.8 x 10^4 CFU/cm^2 at Site 8 (October 2005) while one species of pennate diatom, enumerated at 30 CFU/cm^2 in February, was isolated at the outflow basin.

Overall, the majority of strains cultured from PS deeps were found to be PNSB able to tolerate the chosen H_2S concentration of 0.35 g/l. Aside from two isolates enriched from the Site 7 purple mat and the Site 9 cave (*Thiocapsa*- and *Chlorobium*-like organisms; see following section), no other true purple or green sulfur bacteria were obtained.

By combining and comparing enumeration data from all 12 sites, the mean CFU/cm^2 for Bchl *a*-containing isolates was found lower from sites located on pools versus site within streams by factors of 14 for PNSB and 230 for PSB. It was thought that more turbulent stream sites may receive higher oxygenation thus deterring anoxygenic photosynthesis, but these factors of difference were not great enough to be statistically significant suggesting that running water may not affect oxygen permeation

through the mat. Mean CFU/cm^2 of AAP isolated at each site type was also not significantly different at ~13 times more abundant in pool sites than in steam sites.

3.1.5 Diversity of Isolated Strains

AAP isolated on RO included coccoid to ovoid morphology (strains BF3, BF6, BF8), short straight rods (BF7), curved or bent rods (BF10, BF15, BF60) and long, filament-like rods (BF61, BF62). The color of colonies was most frequently medium to dark orange, accounting for the vast majority of the Bchl *a*-containing isolates. These aerobes were found at every sampling site excluding Site 9 and accounted for 2.6% to 52.4% (February 2005 and March 2006, respectively) of total Bchl-containing colonies. Pale pink strains were cultured in low numbers, for example BF60 (Fig. 3R) at 10 CFU/cm^2 from the basin floating mat (February 2005) and BF15 (Fig. 3S) at 100 CFU/cm^2 from the submerged orange-colored mat at Site 4 (February 2005).

Isolated AAP formed at least three groups defined by their absorption spectrum features. The first major group was represented by strains such as BF6 and BF8, closely related to *Porphyrobacter tepidarius* (97% 16S rRNA sequence similarity) and *Erythromicrobium ramosum* (98% 16S rRNA sequence similarity), respectively. *In vivo* spectra indicated Bchl *a* absorption peaks at 802-806 and 868–871 nm, characteristic of the LHI complex (Yurkov & Beatty, 1998) (Fig. 5E). No absorption of LHII was observed in either strain, though it has been found in *Porphyrobacter neustonensis* (Hanada *et al.*, 1997) and, in *E. ramosum*, a unique LHII absorbance was actually a genus-defining trait (Yurkov *et al.*, 1994). Ratios of Bchl to carotenoids in BF6 and BF8 typically ranged from 2.9 to 3.5 with major carotenoid peaks at 462 nm and 483-90 nm,

Table 5. Phenotypic and phylogenetic diversity of select strains isolated from the Sulphur Mountain thermal spring microbial mats.

Strain	Source Site	Colony Color	*In vivo* carotenoid peaks (nm)	*In vivo* Bchl peaks (nm)	Morphology	Medium of isolation*	Closest 16S rRNA relative	% relatedness
BF8	N3 cyanobacterial mat	orange	423, 462, 479	808, 867	ovoid to short rod	RO	*Erythromicrobium ramosum*	98.9
BF12	N3 cyanobacterial mat	dark pink	465, 500	801, 860, 879	ovoid	PNS	*Rhodopsuedomonas capsulate*	98.0
BF24	N3 cyanobacterial mat	pink-red	415, 461, 482, 508	803, 859, 873	long rods, near spirriloid	PNS	not sequenced	n/a
BF14	N9 subsurface cyanobacterial mat	burgandy	456, 489, 525	803, 887, 904	ovoid-teardrop bud & thin stalk	PNS	*Rhodomicrobium vannielii*	98.6
BF29	N9 subsurface cyanobacterial mat	green	439, 458, 505	641, 751	coccoid in chains up to 8 cells	PS	not sequenced	n/a
BF11	N10 *Thiothrix* mat	deep red	466, 500, 532	808, 877	ovoid to bent rods	PS	*Rhodopsuedomonas palustris*	99.0
BF9	N8 floating cyanobacterial mat	dark pink	452, 478, 510	801, 856, 869	ovoid with single inclusion	RO	*Rhodobacter capsulatus*	98.0
BF25	N8 floating cyanobacterial mat	light pink	457, 481, 511	803, 859, 869	short to long, almost filamentous rods	PNS	not sequenced	n/a
BF28	N7 subsurface purple mat	red-purple	456, 488, 523	802, 857, 874	ovoid with multiple bright inclusions	PS	not sequenced	n/a
BF5	N7 subsurface purple mat	burgundy	463, 494, 528	808, 877	long, bent aggregating rods	PMS	*Rhodopsuedomonas palustris*	99.9
BF48	N7 subsurface purple mat	brown-red	449, 476, 509	800, 859, 867	ovoid to short rod	PNS	*Rubrivivax gelatinosus*	99.9
BF4	N4 pool surface	yellow-brown	412, 465, 538	802, 858	long, thin rods, near spirriloid	PNS	*Brevundimonas diminuta*	99.1
BF6	N4 pool surface	orange	420, 470, 487	804, 869	coccoid to ovoid	RO	*Porphyrobacter tepidarius*	99.0
BF15	N4 pool surface	light pink	475, 499, 534	799, 868, 873	ovoid-rod to pleomorphic	PE	*Paracraurococcus ruber*	93.7
BF16	N4 pool surface	orange-brown	461, 488, 520	801, 868, 889	ovoid-teardrop bud & thin stalk	PMS	*Rhodomicrobium vannielii*	99.9

RO, rich organic medium; PNS, purple and green nonsulfur bacteria selective medium; PS, purple and green sulfur bacteria selective medium, PMS, pyruvate minimal salts medium; PE, *Chloroflexus* medium; n/a, not applicable

as seen in *Por. tepidarius* (Fig. 5E). A second spectral cluster was represented by pink strains BF15 and BF60, related to *Paracraurococcus ruber* (93.7% 16S rRNA sequence similarity). *In vivo* LHI complex absorbed maximally at 868 nm with RC near 801 nm and spheroidene carotenoids predominant near 482, 511 and 540 nm (Fig. 5F). Carotenoic acids and spirilloxanthin are found in BF15's closest relative *Pcr. ruber* (Saitoh *et al.*, 1998). The third grouping for AAP spectra was represented solely by the strain BF4 in which LHI absorbs at 802 and 858-9 nm (not shown). Carotenoid peaks were observed at roughly 30 times the absorption values Bchl *a* peaks, with maxima at 412, 478, 538, 584 and 635 nm. Interestingly, BF4's closest phylogenetic relative is the non-phototrophic, non carotenoid-producing *Brevundimonas diminuta*, while *B. vesicularis* is known to variably produce an orange pigment (Segers *et al.*, 1994). Also in Fig. 5, is the spectral profile of an orange ovoid strain BF62 exhibiting incredibly low amounts of Bchl (in LHI at 870 nm), typical of most AAP (Fig. 5G). Related, is the second interesting feature of the sharp peak at 421 nm. While certain carotenoids can absorb near 420 nm e.g. ζ-carotene at 422 nm or neurosporene at 414 nm (Britton *et al.*, 1977), it is possible the peak may have been the Bchl precursor Mg-protoporphyrin (normally absorbing near 416 nm), which may lend insight on the small amount of Bchl *a* produced (Ouchane *et al.*, 2004). Further study will be required to indentify the compound and learn what conditions either consistently induce carotenoid synthesis or interrupt the Bchl synthesis pathway in such a manner.

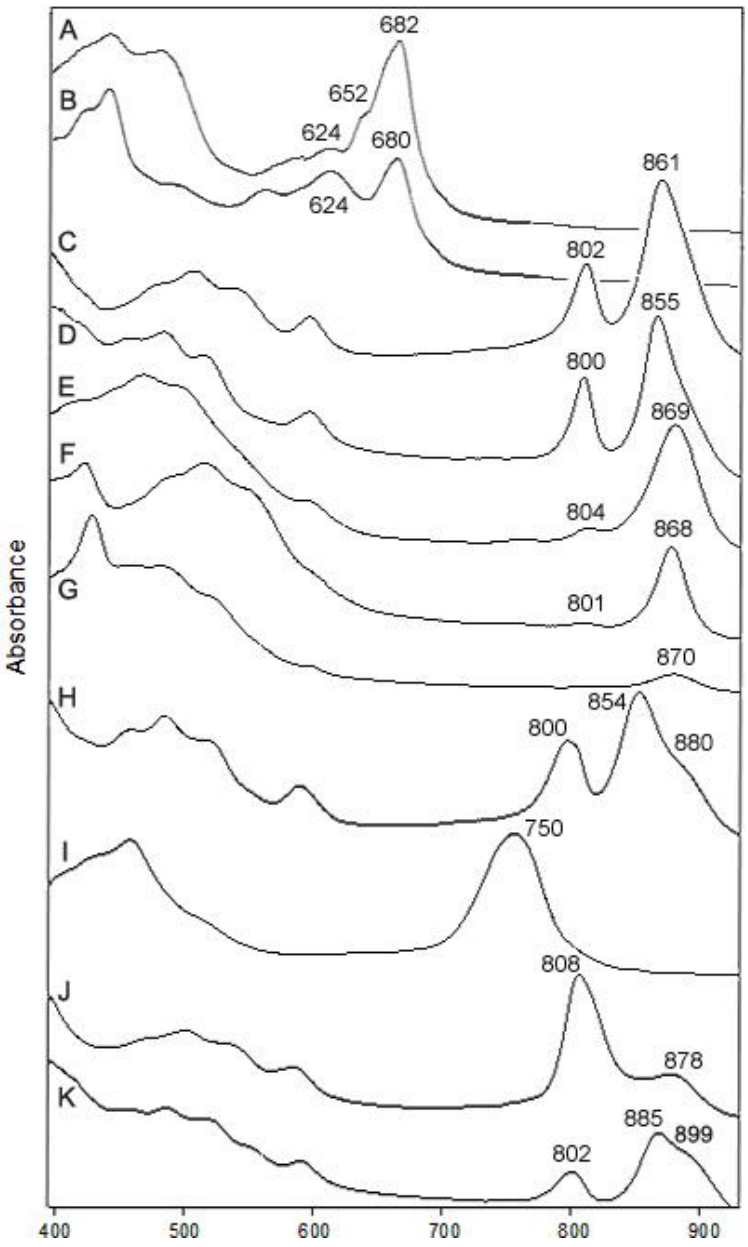

Fig. 5. Spectral diversity from Banff spring isolates including oxygenic strains A) LCB5, *Chlorococcum* and B) Site 11 *Spirulina*; purple nonsulfur bacteria C) BF49, *Rubrivivax* and D) BF12, *Rhodopseudomonas capsulata*; aerobic anoxygenic phototrophs E) BF6, *Porphyrobacter tepidarius*; F) BF15, *Paracraurococcus ruber* and G) BF62, unknown AAP; purple sulfur H) BF28, *Thiocapsa;* green sulfur I) BF27, *Chlorobium* and purple nonsulfur J) BF20, *Rhodopseudomonas palustris* and K) BF14, *Rhodomicrobium vannielii*.

PNSB were the dominant phototrophic isolates with up to a 100% of pigmented cells containing Bchl *a*, nearly all of which were red, purple, pink or olive green in appearance. Most typical morphologies were observed including ovoid (strain BF49; 99.9% 16S rRNA sequence similarity to *Rubrivivax gelatinosus*), short, curved to long rods (BF9, BF37, BF38); teardrop-shaped (BF44) and pleomorphic rods often with tapered ends (BF23, BF33). The most abundant and widespread isolates (e.g. BF9, BF12, BF39) were morphologically and spectrally similar to *Rhodobacter* with absorption peaks at 871, 855 and 800 nm and carotenoids absorbing near 590, 509 and 477 nm, indicating those of the spheroidene series. Within agar deeps, colonies grew purple-pink in the upper oxygenated zone and olive-brown in the lower anoxic area as is typical of *Rba. sphaeroides* in which spheroidene and hydroxyspheroidene are converted to their corresponding ketocarotenoids under oxic conditions changing from brown to red (Imhoff, 2005c). Subsequent 16S rRNA sequencing indicated *Rhodopseudomonas* species also being present e.g. BF5, BF30 and BF 34 each of 99.6% 16S rRNA similarity to *Rps. palustris* (Fig. 3M). Including other pink-red community members of similar morphology (e.g. BF39, BF51), these organisms were ubiquitous at sampling sites including *Thiothrix* mats (from 10 to 3.1 x 10^3 CFU/ml over the year) with greatest numbers cultivated from Site 13 (3.9 x 10^4 CFU/ml in February) and Site 7 (in winter from 3.0 x 10^3 to 1.0 x 10^4). As alluded to above, unique strains represented by BF1, BF13, BF14 and BF16 (Section 3.2), morphologically resembling *Rhodomicrobium vannielii*, were isolated from Sites 4, 6, 7, 8, 9, 10 and 13. As seen in BF1 (Fig. 3Q) the strains exhibited typical ovoid to elongate-ovoid cell morphology and produced exospores, though budding cell length was often up to 0.3 μm longer than the

characteristic 2.0-2.8 μm range. BF14 was spectrally unique exhibiting a LHI (or possibly new complex) absorption maxima at 802 and 885 nm (Fig. 5K), distinguishing it from the LHI in *Rmi. vannielii* which absorbs at 800-807 and 869-872 nm (Imhoff, 2005b).

One of the most dominant morphotypes and representative of a second PNS spectral group were the short rod to spirilloid strains (e.g. BF18, 99% 16S rRNA sequence similarity to *Rps. palustris*; Fig. 3M) isolated from both PS agar deeps and PMS-containing Balch tubes. Purified anaerobically on PMS plates, these strains produce large amounts of Bchl relative to carotenoids (ratio 1:1.7) where, as previously observed by Evans *et al*. (2005), the photosynthetic RC was the dominant complex with Bchl *a* peak maxima near 808 nm at absorbance values over four times those of the LHI peak at 878 nm (Fig. 4J). The LHII peak appeared as a subtle shoulder to the left of LHI near 860 nm.

Another of the most prevalent morphotypes was represented by strain BF9, an ovoid to short curved rod (Fig. 3L) that grows colorless in the presence of oxygen, purple-pink microaerophilically and olive-brown in completely anoxic conditions. Spectral analysis showed LHII peak dominance at 857-860 nm, RC at 800-802 nm and LHI from 869-875 nm. Carotenoids of the spheroidene series were the major pigments and, unlike the previous spectral group, were produced in only about a 1:2 absorption value ratio to Bchl *a*. 16S rRNA sequencing confirmed relatedness to *Rhodobacter capsulatus* at 98.0%. This morphotype was also found at nearly all C&B and UMS sites in every season.

Green and purple sulfur bacterial isolates included only *Chlorobium*-like (BF27; Fig. 3V and Fig. 5I) and *Thiocapsa*-like (BF28; Fig. 3U, Fig. 5H) strains from Sites 9 and 7, respectively. Strain BF27 was a strictly anaerobic, chain-forming coccoid to ovoid organism of a deep grass green color in liquid medium. Spectrophotometric analysis indicated a very strong presence of Bchl *c* at 750 nm and the major carotenoid chlorobactene at 457 nm, produced in a nearly 1:1 ratio (Fig. 5I). Both pigments were typical of the green sulfur *Chlorobium limicola* (Pfennig, 1989). Cell size ranged from 0.4-0.8 μm in diameter and up to 1.3 μm in length, also similar to *Chl. limicola*. The strain exhibited phototaxis in its visibly denser growth along the illuminated side of the Balch tube in conjunction with buoyancy regulation, growing throughout the medium column. This was interesting in that only two of eight known *Chlorobium* species contain gas vesicles (*Chl. clathratiforme* and *Chl. luteolum*) (Imhoff, 2003). Also typical of the family was its ability to grow at low sunlight intensities, measured at Site 9 to average less than 1.3 $\mu E\ m^{-2}\ s^{-1}$. The *Thiocapsa*–like BF28 represented a fifth spectral group. *In vivo* carotenoid peaks were at 460, 487 and 516 nm, similar to the recently described *Thiocapsa imhoffii* (Asao *et al.*, 2007), suggesting spirilloxanthin as the major pigment and an absence of okenone (Fig. 5H). RC and LHII peaks were similar to those of *Thc. roseopersicina* at 800 nm and 854 nm with LHI shoulder at 880 nm, just blue-shifted of that described for *Thc. litoralis* (Puchkova *et al.*, 2000). Morphologically BF28 most resembles *Thc. imhoffii*, though was not observed to form the characteristic tetrads (Fig. 3U).

Cyanobacterial strains of *Phormidium*, *Oscillatoria* and *Anabaena* were obtained on BG11 plates. Dark green single-celled oxygenic phototrophs were isolated from both

the aerobic and anaerobic zones of agar deeps inoculated with samples from Sites 3, 4, 6, 8 and 12. These dominant organisms were morphologically similar to *Aphanothece, Synechocystis, Chlorococcum* (Fig. 3G) and *Dermocarpa* (Fig. 3I). The dominant cyanobacterial morphotype resembling *Spirulina* (Fig. 3F) proved to be atypical regarding the difficulty of its culturing. Contrary to approaches and media published for *Spirulina* and *Arthrospira* cultivation (Ananyev *et al.*, 2008; Cohen *et al.*, 1987; Lu & Vonshak, 2002) the species at Banff springs preferred neutral over basic pH and perished immediately in our variations (pH 8.5-9.5) of the widely used Zarrouk medium.

Spectral analysis of oxygenic phototrophs yielded three basic varieties of absorption profiles, each of which displayed characteristic peaks of Chl *a* near 420 and 440 nm and in the 670-683 nm range (Gitelson *et al.*, 1996). The first group was obtained from single-celled isolates (Fig. 5A) and included *in vivo* cytochrome peaks at 414-416 nm, peaks in the 470-482 nm range, indicating fucoxanthin; the 581-597 nm phycobilin range, phycocyanin near 624 nm, Chl *b* or allophycocyanin at 650-653 nm and a disproportionately large Chl *a* peak at 682-683 nm (Lemasson *et al.*, 1973). The second group (Fig. 5B) was obtained for *Spirulina*-like strains and included large, near-equal peaks of Chl *a* at 680 nm and of phycocyanin at 624 nm (Graham & Mitchell, 1999). *Anabaena*-like morphotypes included first group characteristics and an additional strong absorption near 569 nm in the phycobilin range (Gitelson *et al.*, 1996). The single diatom strain exhibited peaks at 491 and 581 nm, indicative of fucoxanthin; near 636 nm, suggesting phycocyanin or Chl *c* and a large Chl *a* peak at 682 nm.

3.1.6 Microbial Carbon Fixation Rates

Fixation of C_i was measured during five sampling trips (May 2005 and 2007, October, March and November) to estimate the amount of organic inputs facilitated by the microbial community. There were five components to the quantitative measure of autotrophy in the mat: fixation by chemolithoautotrophs measure in an aluminum foil-wrapped "dark" vial, fixation by all community members excluding PSII-containing oxygenic phototrophic organisms (via a light-exposed vial containing the PSII inhibitor diuron), organic production by Bchl-containing phototrophs (diuron minus the dark vial values), production solely by PSII-containing oxygenic phototrophic organisms (light vial values less the sum of dark and diuron fixation) and total community fixation (light-exposed vial) (Steemann Nielsen, 1952). The results from select fractions of the microbial community (dark, oxygenic and anoxygenic productivity) are presented in Table 6 as mg of inorganic C fixed per cm^2 of mat over the 24 h incubation period. The Total column of Table 3 is the value obtained from the mean of the replicate from the light-exposed value and is therefore not the sum of the other fractions of the community, which are based upon readings from separate vials.

Table 6. Productivity of different fractions of the microbial mat community and total carbon fixation for sampling locations at the Sulphur Mountain springs in late spring, autumn and winter.

Site	Date	Type of C-fixation (mg C/cm^2/24 h)			
		Dark	Oxygenic	Anoxygenic	Total
3	May '05	3.44	70.13	0.29	77.32
	Oct '05	3.67	26.69	0.01	33.95
	Mar '06	4.39	57.65	0	64.21
	May '07	13.8	155.19	0	176.92
	Nov '07	6.16	19.78	0	31.06
5, 6	May '05	8.20	27.47	6.39	50.25
	May '07	8.93	0	8.31	14.13
	Nov '07	8.31	10.74	0	23.68
7, 8	May '05	4.21	8.55	3.00	19.96
	Oct '05	13.08	0	0	14.18
	Mar '06	2.77	6.73	0.42	13.67
	May '07	8.22	19.33	2.88	38.66
	Nov '07	11.85	0	0	12.57
9	May '05	1.17	0.40	0	1.19
	Oct '05	1.03	0.12	0.01	1.22
	Mar '06	1.07	0.64	0.03	2.69
	May '07	10.07	2.14	0.17	14.11
	Nov '07	9.07	0.45	0	11.60
10, 11	May '05	1.88	41.71	0.62	46.10
	Oct '05	2.31	1.76	0	5.48
	Mar '06	0.93	6.64	1.60	10.11
	May '07	3.84	18.56	0.70	26.93
	Nov '07	6.21	7.97	0	16.82
12	May '05	0.91	31.77	0.88	34.48
	Oct '05	3.88	4.56	18.85	19.41
	Mar '06	2.84	15.55	0	20.70
	May '07	3.74	50.32	6.81	64.60
	Nov '07	8.72	3.91	0	19.75

At each sampling trip, total community fixation was measured to be greatest at Site 3 *Oscillatoria*- and *Spirulina*-dominated floating mat (Fig. 6). Greatest productivity occurred in May 2007 at 177 mg C/cm^2/day. The May 2007 experiment yielded highest rates at all sites except for the May 2005 samplings at Site 5/6 (27.47 mg C/cm^2/day) and Site 10/11 (41.71 mg C/cm^2/day), with production values dominated by oxygenic phototrophs. Primary production was driven by Chl-containing organisms at most sites

studied accounting for 20%-92% of total production. Exceptions were observed at the Site 12 *Thiothrix-Oscillatoria* mixed mat in November 2007 with over 40% of measured productivity accounted for by non-light-driven fixation (8.72 of 19.75 mg C/cm^2/day). A possible outlier at Site 12 was the anoxygenic fixation value obtained in October of 18.9 mg C/cm^2/day, accounting for 97% of the total. The GSB- and *Beggiatoa*-dominated mat of Site 9 showed insignificant contributions (0.12 to 2.14 mg C/cm^2/day) by oxygenic phototrophic community members in all seasons except May 2007 comprising roughly 15% of a low total fixation. Productivity in the dark accounted for 40% to over 98% of the total activity at this shaded cave site, though anoxygenic phototrophic fraction values did not fluctuate in any correlative manner. This may suggest that chemoautotrophy was the dominant metabolic scheme of the *Phormidium*- and *Chlorobiaceae*-like organisms observed at this site. Overall, the values obtained (Table 6) generally reflected the relationship of higher average temperatures and greater light availability to productivity. The seasonal pattern for all sites was similar at both spring groups with higher values in each May, facilitated by the increase in daylight and temperature. Summer month values may have been comparatively lower to those found in May due to the increased biomass of the mat containing older and less active, or dead inactive cells trapped within the EPS. While light and heat energy became increasingly available in the summertime, self-shading and aging portions of the community may have contributed to lower fixation rates per unit area. Change in dark fixation was generally insignificant at all sites except the combined Site 7/8 location in May and October 2005 and March.

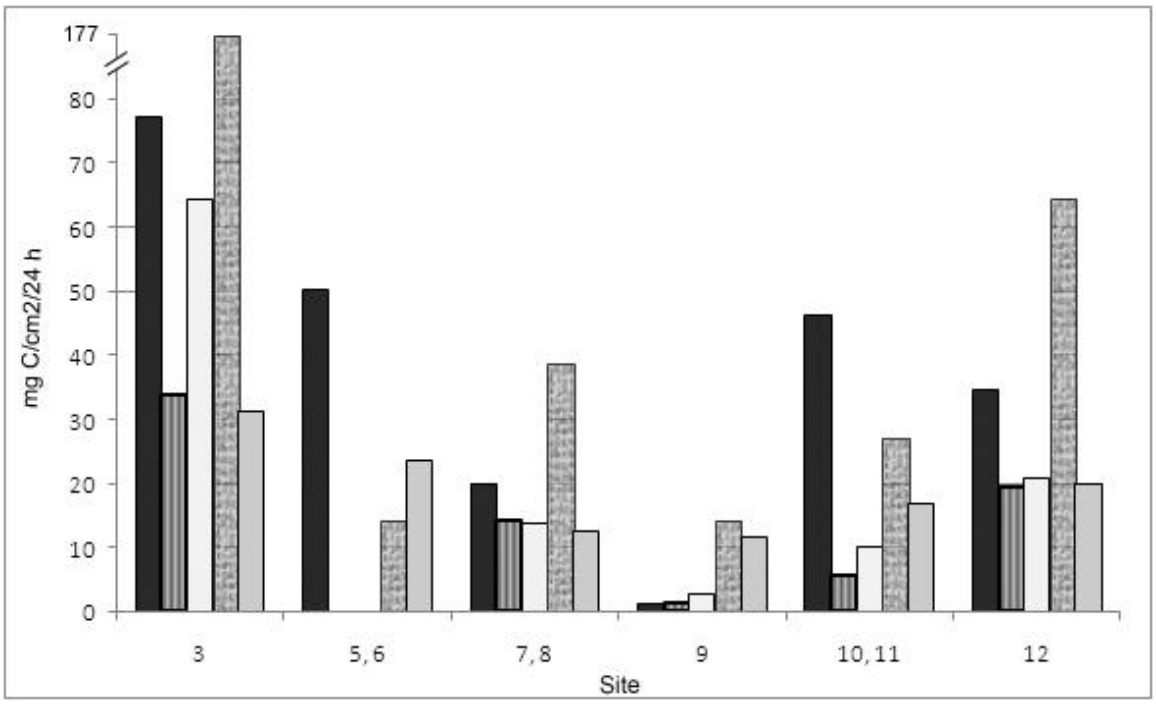

Fig. 6. Seasonal comparison of total community productivity values (mg C fixed per cm^2 per 24 h) for all sampling sites. Black column, May 2005; lined, October 2005; white, March 2006; textured, May 2007 and grey, November 2007. No sampling done at 5, 6 in October or March.

Evident at Site 5, 6- the mat that had vanished during the late summer of 2005- were signs of microbial succession or, change in abundance of bacterial populations from relatively few dominant pioneering genera to many. The predominance of dark fixation in the following months, accounting for ~63% of total fixation in May, could have reflected re-establishment of the mat initially by *Thiothrix* and other chemoautotrophs, as oxygenic fixation was not measured at significant rates. Colonization was followed by an increasing oxygenic phototrophic presence accounting for almost 11 mg C/cm^2/day of the total 23.7 mg C/cm^2/day measured, compared to 8.3 mg C/cm^2/day obtained by dark fixation.

Compared to other studies of spring mat systems, the results obtained for the Banff communities indicated exceptional productivity. A study of the *Microcoleus* and *Lyngbya*-dominated marine mats of Shark Bay Western Australia measured fixation rates as high as 1.47 mg C/cm^2/day, to which results here comparatively range 3.7 to 120 times larger (Bauld, 1984). Namsaraev et al. studied the *Phormidium*-, algae- and *Chloroflexus*-dominated mats of the alkaline Bol'sherechenskii hot (>60 °C) springs finding a maximum total community production of 1.3 g C/m^2/day and a highest dark fixation rate of 0.806 g C/m^2/day (Namsaraev *et al.*, 2003). Converting units of area, even the lowest total community results at Banff springs exceeded the Bol'sherechenskii maximum by 9 times while the Site 3 May 2007 result of 177 mg C/cm^2/day was over 1300 times greater. The maximum dark fixation at Banff occurred in May 2007 at 13.8 mg C/cm^2/day, or 17 times greater. Interestingly, compared to a study of pelagic microbial dark production in a shallow sulfidic estuary of Ebro River, the results obtained here for dark fixation ranged from 217 to over 3200 times the marine values (Casamayor *et al.*, 2001). Oxygenic phototroph production rates from the Urinskii alkaline hot springs were found to be an average of 2.1 g C/m^2/day (0.21 mg C/cm^2/day), observed at the 45-50 °C zone where *Oscillatoria limosa* and diatoms were predominant (Bryanskaya *et al.*, 2006). A maximum anoxygenic phototroph fixation value of 0.42 g C/m^2/day was found in the 35-40 °C zones where *Chloroflexus* was dominant, though this value is roughly 70 times less than the maximum observed at the PNS- and PSB-dominated Banff Site 7.

3.1.7 Microbial Dynamics and Physella johnsoni trends

Our working hypotheses included that 1) there would exist a correlation between the development of the cyanobacterial mat and number of *P. johnsoni* at that site. As primary producers of organic C, microbial photoautotrophs should form the base trophic layer in the springs. It was not certain if numbers of anoxygenic phototrophic bacteria per cm^2 of mat would be correlated to snail fluctuations as it was uncertain whether expanse of the mat and density of anoxygenic phototrophs within it were directly proportional. A phototroph-snail relationship would have potentially manifested as either simultaneous high counts in each or a relatively high development of phototrophs preceding an increase in *P. johnsoni*. 2) There may not be a correlation between *P. johnsoni* and colorless bacteria unless one is determined to exist between heterotrophs, sulfate reducing bacteria, etc. and Chl- and Bchl *a*-containing strains. As mentioned, it has been shown that proliferation of heterotrophic bacteria often depends upon exopolymeric substances excreted by algae for a carbon source (Romani & Sabater, 1999) and heterotrophs may also benefit from the phosphatase activity of neighboring photosynthetic organisms to uptake phosphorous (Espeland & Wetzel, 2001). If such is the case, a similar fluctuation between populations of strict organotrophs and phototrophic bacteria should occur. Conversely, opposite population trends may be expected if grazer consumption of Chl- and Bchl-containing organisms was so stark that it improved the competitive capabilities of other bacteria and, in turn, their numbers. Grazer impact on periphyton biomass and composition has been well-documented (Danger *et al.*, 2008; Kawata *et al.*, 2001; King-Lotufo *et al.*, 2002). If such a grazer-induced impact occurred at Banff, it may be due to increased access to detrital or water

column-based nutrients or snail mucous trails as a carbon source and fecal matter as a reservoir of phosphorous (Liess & Haglund, 2007), benefitting both aerobic and anaerobic anoxygenic phototrophs, which are primarily heterotrophic. It could therefore be assumed that carbon inputs from grazers that would benefit colorless organotrophs could also support photoheterotrophs. 3) Photosynthetic activity (primary organic production), a direct quantification of the organics available to higher trophic levels, should correlate with the fluctuation in *P. johnsoni* enumerated (King-Lotufo *et al.*, 2002). Note that while grazers tend to have impacts on periphyton biomass and taxonomic structure (over 3/4 of the time), they have a lower tendency to significantly affect overall productivity of the mat, or less than 30% of the 89 experiments as reviewed by Feminella (1995). That said, while general and significant trends appear throughout the literature, studies of interactions between prokaryotes and eukaryotic microbes have often proved to be idiosyncratic descriptions, unique to the particular niche and species under observation.

The limited direct observations over past years suggested roughly a 30% to 60% difference in the sheer area covered by the mat at each of the C&B and UMS spring systems from winter to late spring. Mat thickness was variable throughout all seasons and rose dramatically in August at select sites, for example, a tripling in some areas of the Site 3 floating Basin mat between May and August 2007. The microbial mat, along with rocks, twigs and other detrital matter provides a structural support system for *P. johnsoni*. The Fig. 7A photograph shows typical *P. johnsoni* orientation on the greener portions of

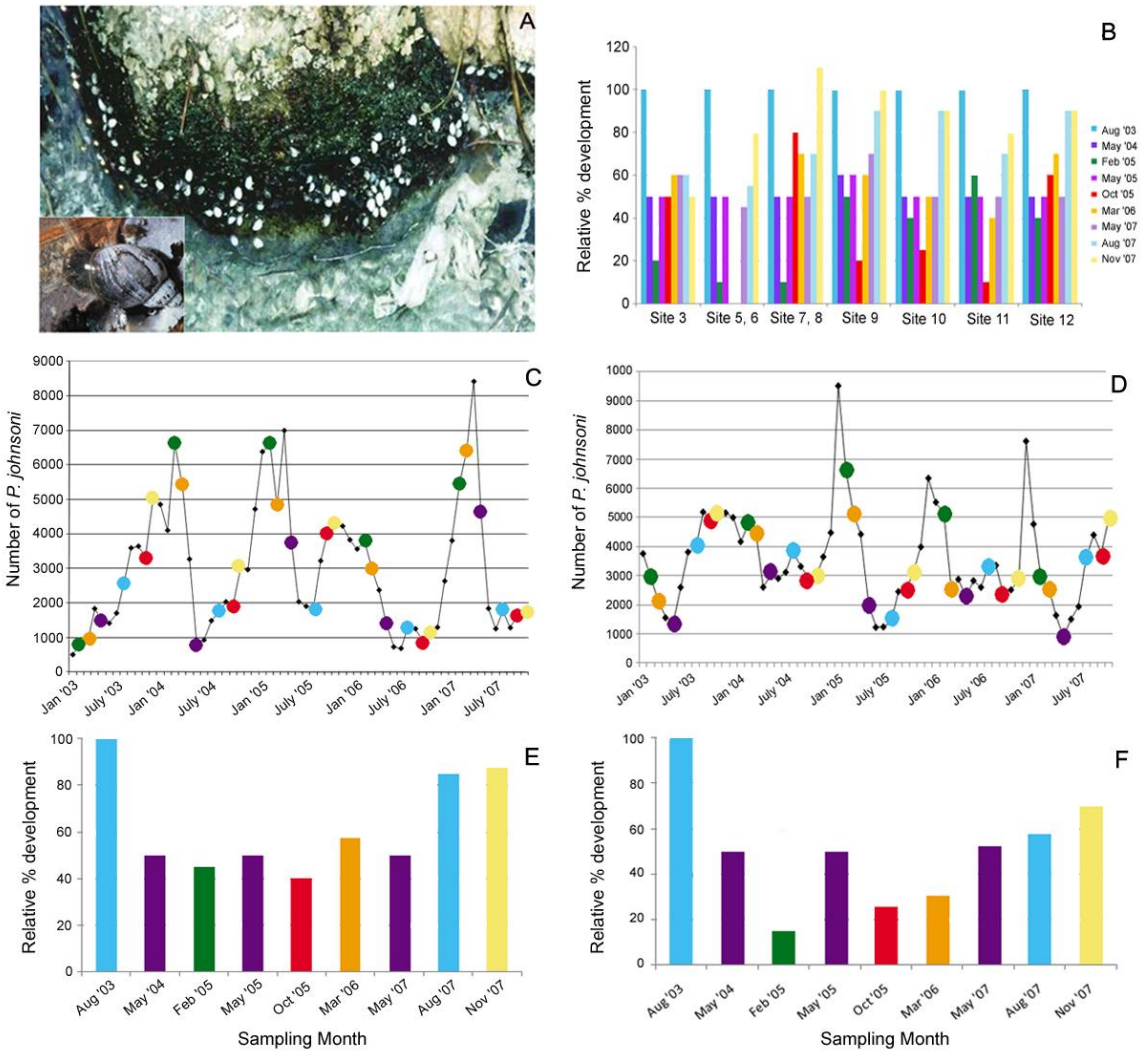

Fig. 7. *Physella johnsoni* and changes in mat area. A) Photograph of *P. johnsoni* on the surface of cyanobacterial mat growth at the UMS. Inset: Close-up of the snail. B) Relative percent development of sites harboring snails compared to the maximum areal expanse (i.e. 100%) observed in August 2003. C) Sampling months color-coded and superimposed on a graph of monthly snail counts at Sites 10, 11 and 12 combined. Colors correspond to sampling months on the X-axis of Panel E. D) Sampling months color-coded atop and superimposed on a graph of monthly snail counts at Sites 3, 5 and 6 combined. Colors correspond to sampling months on the X-axis of Panel F. E) Bar chart of the averaged relative percent development for Sites 10 to 12. F) Bar chart of the averaged relative percent development for Sites 3, 5 and 6 (due to loss of locations 5 and 6, October and March values are Site 3 only).

the mat; snails clinging to the epilithon to avoid getting swept away by the outflow stream. An increase in mat expanse should provide increased habitat, though there was clearly a lag effect as largest mat development and highest snail counts seem to follow inverse trends. Fig. 7 displays this incongruence with monthly *P. johnsoni* counts compared to the areal developments of Sites 10-12 combined (Fig. 7C) and Sites 3, 5 and 6 (Fig. 7D) combined, which were expressed as a relative percentage of the maximum expanse observed in August 2003 (as explained for Table 1). Highest snail counts were generally obtained in February or March at Sites 10-12 and November or December for 3, 5 and 6, and these were compared to observations of mat development. Sampling months are shown color-coded in Figs. 7E and 7F and correspond to the color indicating the same month of *P. johnsoni* enumeration in Figs. 7C and 7D. Despite discontinuous observations, it still was apparent that larger mat expanses in the summertime often coincided with the trough of snail fluctuations, while larger snail populations and smaller mat area co-occurred in the winter. Common in ecology, the temporal mismatch between predator/grazer population responses to their variable resources has been referred to as "tracking inertia" (Solbreck & Sillen-Tullberg, 1986). From about mid-October, when mat productivity and area decrease, snails may still increase in population at a similar rate. Overconsumption of available resources may cause the beginnings of the sharp decline witnessed in February or March. The recovery of the microbial mat in spring and typical maximum development in summer would thus facilitate the response in *P. johnsoni* by providing increased organics in the form of exopolymers and bacterial cells themselves. Larger mat areas also provide greater space for reproduction and the avoidance of energy expenditures on interference behaviors, e.g. shaking shells after

contact with another individual, as found in *Physella virgata* (King-Lotufo *et al.*, 2002). As previously mentioned, it has also been observed in *P. virgata* and others that high snail density tended to depress grazing rates and, in turn, growth rates (Feminella, 1995; King-Lotufo *et al.*, 2002). A highly dense population may inevitably result in a stark crash as many specimens would not attain sufficient growth/maturity to reproduce, while those snails that were able to also decline as *P. johnsoni* are semelparous, rearing offspring only once before dying shortly thereafter. The hatching of young molluscs may then coincide with a seasonal decline/sloughing phase of the mat thus creating the temporal mismatch suggested by Fig. 7.

The near complete loss of several microbial sampling sites at the LCB in mid 2005 to early 2006 corresponded to record breaking rainfall observed in August 2004 and June and September 2005. An effect was also witnessed on the LCB snail population: from 2852 snails counted in February 2005, numbers plummeted to 40 specimens in 5 months and taking 8 months to recover to 1592 (Lepitzki, 2007). Mat expanse and snail numbers at the UMS Sites 10, 11 and 12 decreased drastically suggesting a connection to the excessive rainfall, the decrease in mat area or likely both.

Calculation of the average number of months between maximum and minimum snail census values at the LCB and two UMS sites (from 2001 to 2007) showed cycles took approximately nine months at combined Site 5/6, just over seven months at Site 10/11, and about six and a half months at Site 12. June had lowest snail enumeration values most frequently. The longer average rise at 5/6 was influenced by the extended recovery period necessary after the 2005 microbial mat habitat loss than to the difference in dominant species at each site, but Site 12 *Thiothrix* could be suggested as influential in

abiding the apparent quicker recovery of *P. johnsoni*. This would also be suggested in 2007 (Lepitzki) when an offshoot of the UCB stream was observed to harbor thick *Thiothrix* mats that were quickly colonized by migrant snails, suggesting a less selective and more opportunistic approach to their food choices. Unfortunately the mat was not observed at its infancy to identify the founding component of the microbial community. At the UMS system it was often seen that below the layers of *Thiothrix* there often was a hidden layer of *Phormidium*. This may suggest that oxygenic phototrophs are the pioneers at these springs providing a scaffold whereby *Thiothrix* can occasionally overtake them.

Fig. 8 shows a comparison of chemolithotrophic and oxygenic photosynthesis-driven carbon fixation at Sites 3, 10/11 and 12 with *P. johnsoni* enumeration data from 24 months over a three year period. As observed for area of mat, maximal total community fixation at Sites 3 (Fig. 8A) and combined Site 10/11 (Fig. 8B) appeared to occur during the periods of lowest snail populations. Site 3 May 2005 and 2007 values were a fairly clear example in that over twice the productivity (77 to 177 mg C/cm^2/24h) occurred at times when one-third fewer snails (721 down to 526) were present. That large May 2007 value occurred just as the slow increase in *P. johnsoni* numbers began until the last C-fixation measurement in November 2007 revealed incredibly low photosynthetic activity. While it can be postulated that intense grazing pressures could have physically damaged cyanobacteria and the autotrophic capabilities of other mat surface members, C-fixation rates can be thought of in the same way as mat expanse and snail numbers fluctuate. The temporal lag of a population catching up to available resources, the aforementioned

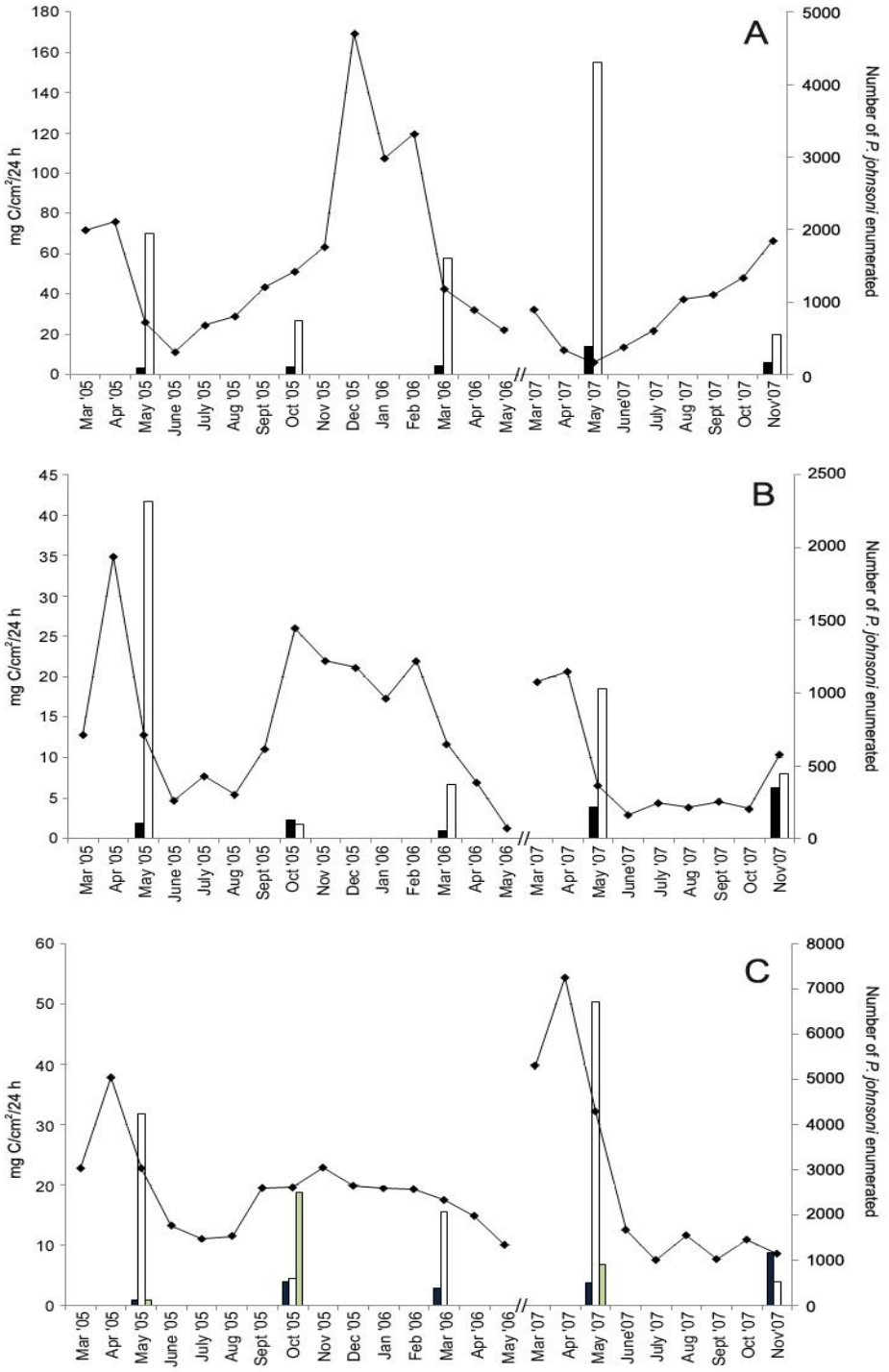

Fig. 8. Photosynthetic activity reflected in mg of carbon fixed per cm^2 of mat over 24 hours (primary axis) compared to monthly *P. johnsoni* counts (secondary axis) for A) Site 3, B) Site 10, 11 and C) Site 12. Black bars, protein production in the dark; white bars, fixation by oxygenic phototrophs; grey bars (in C only), anoxygenic phototrophic fixation; line, *P. johnsoni* enumeration.

tracking inertia, may be inferred by the rudimentary patterns obtained at Sites 3 and 10/11.

Visually, Site 12 (Fig. 8C) does not suggest the mismatch between *P. johnsoni* numbers and production of organics as well as the other two locations. This might have been a result of the mat composition at this site because, as mentioned, the Site 12 mat was observed to be nearly equally dominated by *Thiothrix* and oxygenic phototrophs. While *Thiothrix* does have chemoautotrophic capabilities (which may then explain the incredibly large November 2007 dark-fixation result), the fixation at this site was driven by oxygenic and anoxygenic phototrophs. If large numbers of snails were present and consuming (whether by accident or not) *Thiothrix* more often, the result could then be to exert less grazing pressure or damage on phototrophic and photosynthetic bacteria. If *P. johnsoni* has the capability to affect the mat in a G → P (Section 1.5.2) manner (Feminella, 1995), it could be lessened.

A comparison of Chl *a* content extracted from Site 3, Sites 10 and 11 combined and Site 12 samples to enumeration of *P. johnsoni* is shown in Fig. 9A, B and C, respectively. While lacking many replicates, the graphs may indicate a rudimentary correlation. As mentioned above, density of nutritive photopigments in prokaryotes is typically highest when day length is shortest and light intensity is lowest i.e. late fall and winter and snail population fluctuated in a similar manner with higher numbers at low-light periods of the year. An increase in the concentration of nutrients results in a decrease in the grazing distance and expenditure of energy necessary for snails to feed to attain reproductive capabilities, but as described above, the main determinant of the spatial dynamics of snail interaction would be mat expanse. Perhaps it is only a spurious

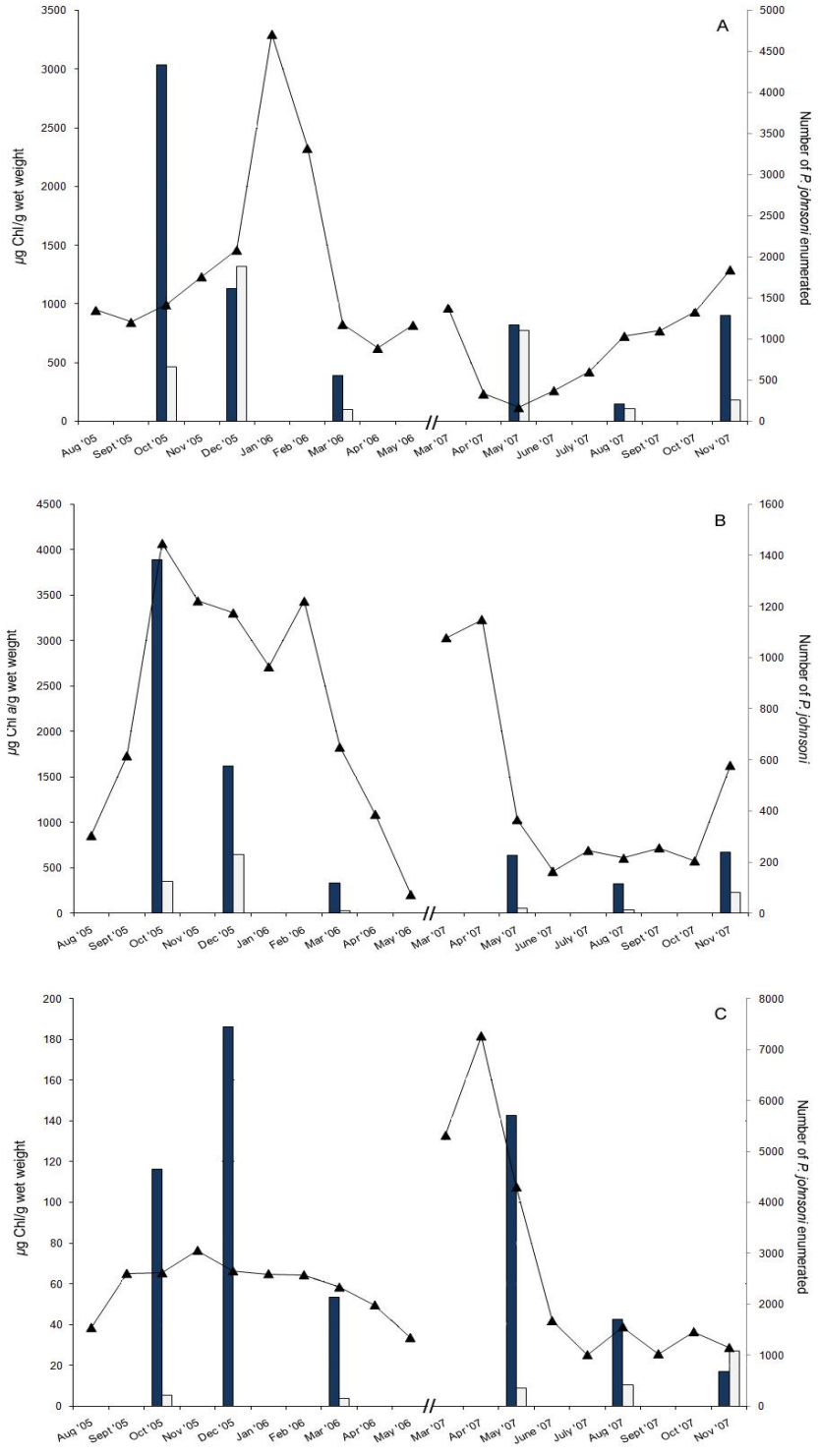

Fig. 9. Chlorophyll *a* and *b* extracted from mat samples in select months compared to monthly enumeration of *P. johnsoni*. A) Data from Site 3, C&B floating mat; B) combined data from Sites 10 and 11 at the UMS and C) Site 12.

relationship between mat photosynthetic pigment density and *P. johnsoni* numbers. A larger sample set would be required to clearly identify mat Chl concentration as a good predictor of snail population.

3.1.8 Closing Comments

In summary, observations of the Sulphur Mountain thermal spring microbial mats showed a diverse matrix of prokaryotic and eukaryotic phototrophs that did not significantly vary in membership over the year, while mat area fluctuated greatly by season. Consistent major mat-forming oxygenic phototrophs included *Phormidium*-, *Spirulina*-, and *Oscillatoria*-like cyanobacteria and single-celled algal species resembling genera *Chlorococcoccum*, *Dermocarpa* and *Aphanothece*. Purple nonsulfur *Rhodobacter*, *Rhodopseudomonas*, *Rhodomicrobium* and *Rubrivivax* were the most commonly isolated photoheterotrophs, followed by aerobic anoxygenic *Erythromicrobium* and *Porphyrobacter* species.

This investigation of Sulphur Mountain springs focused on six microbiological components to compare to the fluctuation of *P. johnsoni*: 1) observed microbial mat expanse, 2) dominant morphotypes observed at different locations in various seasons, 3) chlorophyll content, 4) photosynthetic activity measurements and 5) the numbers of Bchl *a*-containing strains and 6) colorless, heterotrophic bacteria enumerated at each site. Regarding our first working hypothesis, positive correlations between density of Bchl-containing strains and *P. johnsoni* were found at three of five sampling locations, while no relationship was found in the vicinity of the UCB purple mat and a weak negative slope resulted at Site 5/6, likely influenced by the dramatic precipitation and sloughing

events incurred there. The most consistent indicator of snail numbers at the time of sampling appeared to be Chl content per unit mass of the mat. This is logical as photosynthetic activity was at its greatest in warmer months of high insolation (when snails were at their lowest numbers) and two results during these periods would be abundance of exopolymeric substance production (thus decreasing pigment proportion per unit weight) and a decrease in cellular Chl compared to the levels required to harvest fall and winter season luminosity.

Qualitative assessments of microbial proliferation were made visually of the area spanned by the microbial mat at each site relative to the maximum expanse observed in August of 2003. Juxtaposed to *P. johnsoni* population counts from Lepitzki (2002), the trend was that lowest mat development correlated with highest snail counts. This was followed by a severe snail population decrease during mat rebuilding to maximum expanse then an approximate 7-month decline in development while snail numbers grew to their peak. Snail fecundity therefore appeared to be dependent on mat components allowing continued reproduction of *P. johnsoni* during decline of the mat. It is important to acknowledge that what was significant from a statistical perspective, may not reflect what was significant biologically. An approximate 20% difference in grazing area may beg the question of significance, but much of the mat expanse was already unavailable to *P. johnsoni* due to running water, rocks or branches and other obstacles. Any further limit in territory may have contributed to the influences of increased mollusc density that can include physical interference between specimens, as previously referred to in Section 1.5.2. Also noted in 1.5.2, another physid, was the tendency by *P. virgata* to reproduce only after attaining a certain shell diameter and that this threshold(necessary) size

increased in the presence of an environmental stress such as a predator (Crowl & Covich, 1990). This trait extends the risk of fatality by other means before rearing offspring thus emphasizing the importance of suitable grazing environment size to prevent such self-limiting characters. Inertial tracking appears to be the most suitable concept lending insight to the time lag between these grazers and the volume of organics available for their consumption.

Another question was why *P. johnsoni* did not colonize the "control site" within the West Cave since 1) thickness did not differ greatly from other locations (found up to 9 mm; Table 1), 2) the major oxygenic phototrophs were present (*Phormidium*, *Oscillatoria*, *Spirulina*) and 3) the most common of PNSB isolated at other sites (*Rhodomicrobium*, *Rhodopseudomonas*) were also found there. Site 13 was distinct from most other sites in three major ways: the water within the cave was not sulfidic, the microbial population was observed to lack *Thiothrix* and the temperature at the surface of the mat was always 6-8 °C cooler. From what is known about the genus, the presence of sulfide imparts no benefit onto *P. johnsoni* and from previous sections it can be argued that *Thiothrix* was important, but not a sole determining factor as a nutritive source. The most likely factor then was that of a temperature barrier providing insufficient thermal energy for incubation of snail eggs or suitably high rates of mat-facilitated C_i-fixation.

As stated in Section 1.4.1, cyanobacteria serve as primary producers of organics such as glycolate during photorespiration or fermentation products of acetate, ethanol, lactate and other simple compounds and these substrates facilitate the growth of heterotrophic and other bacteria that may be nutritionally significant to *P. johnsoni*. Studies have provided examples of filamentous cyanobacteria themselves being integral

to mollusc diets in some cases, while a preference towards phototrophic eukaryotes, heterotrophic bacteria and detritus occur in others (Sheldon & Walker, 1997). The case for *P. johnsoni* is uncertain. If detritus was a major source of carbon for *P. johnsoni* as in many *Physa* species (Kawata *et al.*, 2001) it still follows that a larger mat area traps more material thus increasing accessibility (Lawrence *et al.*, 2002). As mats at Sites 3, 5/6 and 8 were built upon forest debris, it is likely some detritus consumption occurred while it was observed that snails tended to be positioned directly upon bacterial formations rather than forest litter. Animal byproducts were not likely available in any significant amounts as it has been documented only few terrestrial species have been observed at the springs as predators of *P. johnsoni* (Lepitzki, 2002). Due to strict controls on the availability of test specimens of *P. johnsoni*, an aspect lacking in the study was direct experimentation with the snail in controlled grazing environments as other studies have performed (McCollum *et al.*, 1998; Sharfstein & Steinman, 2001) or gut content analysis. These analyses may provide definite conclusions as to which of the many available organics are foundational in *P. johnsoni* nutrition.

3.2 Rhodomicrobium vangemerdenii sp. nov., an exospore-forming purple nonsulfur bacterium isolated from sulfidic thermal springs in Banff, Alberta, Canada

3.2.1 Abstract and Opening Remarks

During the investigation of the Sulphur Mountain thermal springs in Banff, Canada, three strains of ovoid prosthecate purple nonsulfur bacteria were isolated under

photoheterotrophic conditions. Strains BF1, BF14 and BF16 were Gram-negative, exhibited a vegetative growth cycle, produced motile swarmer cells and exospores, grew optimally photoheterotrophically and were capable of chemoorganotrophic and photolithoautotrophic growth. No growth factors were required. Physiological and phylogenetic analysis showed the strains were related to the genus *Rhodomicrobium* and 16S rRNA sequences matched the type species *R. vannielii* with 99.6% (BF1), 98.6% (BF14) and 99.6% (BF16) similarity. Spectral analysis revealed all strains contained Bchl *a*, β-carotene and carotenoids of the spirilloxanthin series including lycopene and rhodopin. Based on sequence data and phenotypic traits, strains BF1 and BF16 were assigned as new strains to *R. vannielii*. The greater phenotypic dissimilarity of BF14, including novel *in vivo* spectral peaks at 885 nm (LHII) and 900 nm (LHI) and lower DNA sequence similarity warrant its distinction as a new species for which the name *Rhodomicrobium vangemerdenii* was proposed. The type strain is BF-14^T (=DSM 23294^T).

The establishment of the genus *Rhodomicrobium* originally distinguished *R. vannielii* from its budding relatives in *Rhodopseudomonas* (Duchow & Douglas, 1949) with formation of prosthecae and its characteristic growth cycle as the two clearest differentiating traits (Imhoff, 2005b; Whittenbury & Dow, 1977). The designation also maintained phototrophy as an important taxonomic marker to distinguish *R. vannielii* from the hyphae-/prosthecae-forming and budding chemoorganotrophs in *Hyphomicrobium*, now known to be phylogenetic relatives near 90% 16S rRNA sequence similarity (Rainey *et al.*, 1998).

Since Duchow and Douglas' introduction and description of the genus (Duchow & Douglas, 1949), a single species has represented *Rhodomicrobium*. *R. vannielii* has since been isolated from a variety of illuminated habitats including most recently the sediment of a ditch in southwest Germany (Heising & Schink, 1998), a 72-74 ˚C alkaline sulfidic hot spring in south central Russia (Namsaraev *et al.*, 2003), a 1 m subsurface sampling of the 56 ˚C western Malaysian Gadek hot springs (Ainon *et al.*, 2006) and the brackish meromictic Lake Shira in Siberia (Lunina *et al.*, 2007). In the present paper, we describe *Rhodomicrobium* relatives isolated during a population study of the cyanobacteria- and *Thiothrix*-dominated bacterial mats within the Sulphur Mountain Thermal Springs at Banff National Park, Alberta, Canada (51˚ 10' N, 115˚ 35' W).

A polyphasic approach, including 16S rRNA sequence data and the standards recommended for description of novel anoxygenic phototrophs (Imhoff & Caumette, 2004), identified two new strains of the type species and, since 1949, a new species to the genus for which the name *Rhodomicrobium vangemerdenii* is proposed. In a career spanning five decades, Hans van Gemerden contributed extensively to our understanding of purple and green bacterial ecology (van Gemerden, 1983; van Gemerden & Mas, 1995b), metabolism and physiology (Hansen & van Gemerden, 1972; van Gemerden, 1968; van Gemerden & Mas, 1995a), intraspecies interactions (van Gemerden, 1974; van Gemerden & Beeftink, 1981), sulfur cycling (Heijs & van Gemerden, 2000; Jonkers *et al.*, 1998; van Gemerden, 1967) and, in his early research days, plant systems (Bange & van Gemerden, 1963). We propose that the late Van Gemerden be honored for his vast contributions to anoxygenic photosynthesis research with the naming of this new purple bacterial species.

Strain BF14 was isolated from within the dimly lit UMS cave at Site 9, while BF16 was discovered at the C&B Site 4 (the orange *Chloroflexus*-dominated mat growing 30 cm subsurface of Billy's Pool) and BF1 from the LCB Site 6. These isolates were of great interest and grouped separately from other morphotypes due to their distinct resemblance to *Rhodomicrobium vannielii*. As mentioned above in Section 3.1.5, the ubiquity of these morphotypes at the Banff Springs was confirmed by nine sampling trips at 12 locations across the spring system consistently yielding *Rhodomicrobium*-like isolates. All seasons were included and highest enumeration values were obtained in March, August and November at 1000 CFU/cm^2 of microbial mat.

3.2.2 Culture properties

All three strains grew as small 0.5 to 1.5 mm colonies, circular to irregular in form and deep orange-brown to red-purple in color within the anoxic zone of agar deeps incubated in the light. On anaerobic plates, individual colonies were typically less than 1 mm, pulvinate and undulate with pigmentation the same as observed in deeps. Anoxic liquid cultures of BF1 and BF16 formed rusty red suspensions with slight aggregation of cells. Strain BF14 had slightly deeper pigmentation and a strong tendency to aggregate in liquid, resulting in flocculent cultures and settling. Dark aerobic conditions on SMS plates yielded thin colorless growth becoming bright, light orange punctiform colonies typically after about 72 h of growth.

3.2.3 Morphology

Cellular morphology was examined in exponential phase cells grown anaerobically in SMS medium (Fig. 10A-C). Liquid cultures included characteristic almond to ovoid

mother cells producing thin prosthecae terminating in a coccoid bud (Fig. 10C) differentiating into a polyhedral refractive exospore (Fig. 10A) or spherical motile swarmer cell. Late-exponential cells formed 1-2 bright circular inclusions similar to the refractive poly-β-hydroxybutyrate stores observed in other purple nonsulfur strains (Imhoff *et al.*, 2005). BF1 measured 0.8-1.9 µm wide x 1.1-3.2 µm long; BF14, 0.9 -1.3 µm x 1-2.3 µm and BF16, 0.9-1.4 µm x 1.2-2.8 µm, found similar to *R. vannielii*. Prosthecae were formed by all strains and were estimated to be 0.2 to 0.4 µm wide, reaching lengths several times that of mother cells (up to 8 µm). Exospores ranged from 0.8 to 1.3 µm in diameter.

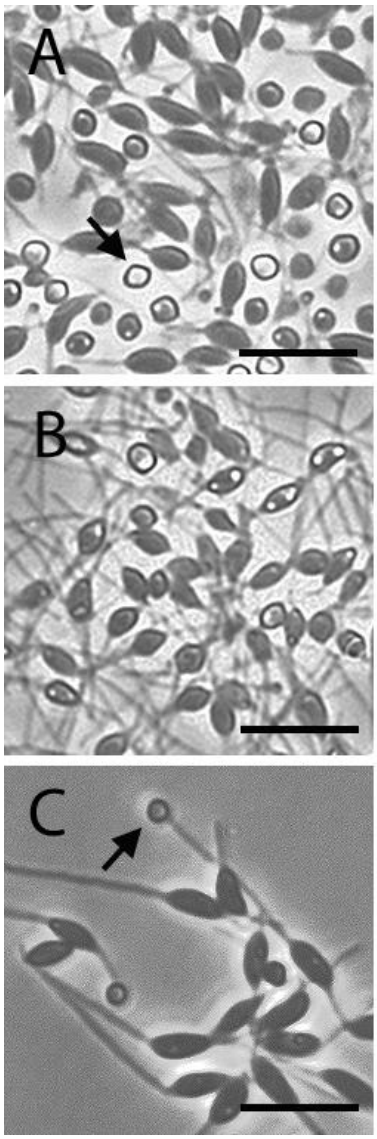

Fig. 10. Phase contrast micrographs on late-log phase photoheterotrophic cultures of A) BF1, B) BF14 and C) BF16. Stages of the vegetative cycle include prosthecated mother cells (dominant in B), refractive exospores (indicated by the arrow in A) and young budding swarmer cells (arrow in C). Bar, 5 µm.

3.2.4 Photosynthetic Pigments and Photosynthesis

Absorption spectra for strains BF1 and BF14 are shown in Fig. 11. BF1 and BF16 had identical *in vivo* and solvent-extracted spectral profiles and showed *in vivo* Bchl *a* major

peaks at 868-872 nm indicating the LHI complex and at 801-803 nm suggesting the LHII complex superimposes the RC 800 nm peak. A red-shifted complex previously unobserved in *R. vannielii* (Heising & Schink, 1998) is apparent as a shoulder at 883-886 nm.

Strain BF14 exhibited a similar RC/LHII maximum at 802-803 nm, but an even greater red-shifted LH complex apparent as a shoulder at 900 nm in addition to the distinct LH complex peak at 883-886 nm. Purification and crystallography of the LH and RC complexes will prove valuable for comparison with the known apparatuses of other purple nonsulfur bacteria. It is likely that new strain BF14 produces an elaborate LH system composed of four different LH complexes. Intricate, multi-LH compositions have been observed previously in *Rhodopseudomonas palustris* (LHI absorbing near 880 nm, LHII at 808 and 862 nm and LHIV at 808 nm) and in *Rhodopseudomonas acidophila* which has LHIII (absorbing 800 to 820 nm) rather than LHIV (Evans *et al.*, 2005; McLuskey *et al.*, 2001). Recently it was shown in *Thermochromatium tepidum* that Ca^{2+} ions were responsible for an unusual Q_y absorption at 915 nm for its LHI (Kimura *et al.*, 2008). As the isolation site of BF14 is an extremely calcium-rich spring, the possibility of an analogous or new conformation is possible.

In vivo carotenoid profiles for each of the three strains included maxima at 458-462, 488-491 and 520-525 nm typical of the spirilloxanthin series including rhodopin (as the major component) and lycopene. The absorption spectrum for pigments extracted in acetone:methanol had maxima at 363, 381, 448, 472, 499, 601 and 769 nm for all strains (Fig. 11). Peaks at 448 nm and 472 nm suggest the presence of β-carotene as noted in the

type species (Heising & Schink, 1998) and the peak at 769 nm confirmed the production of Bchl *a*.

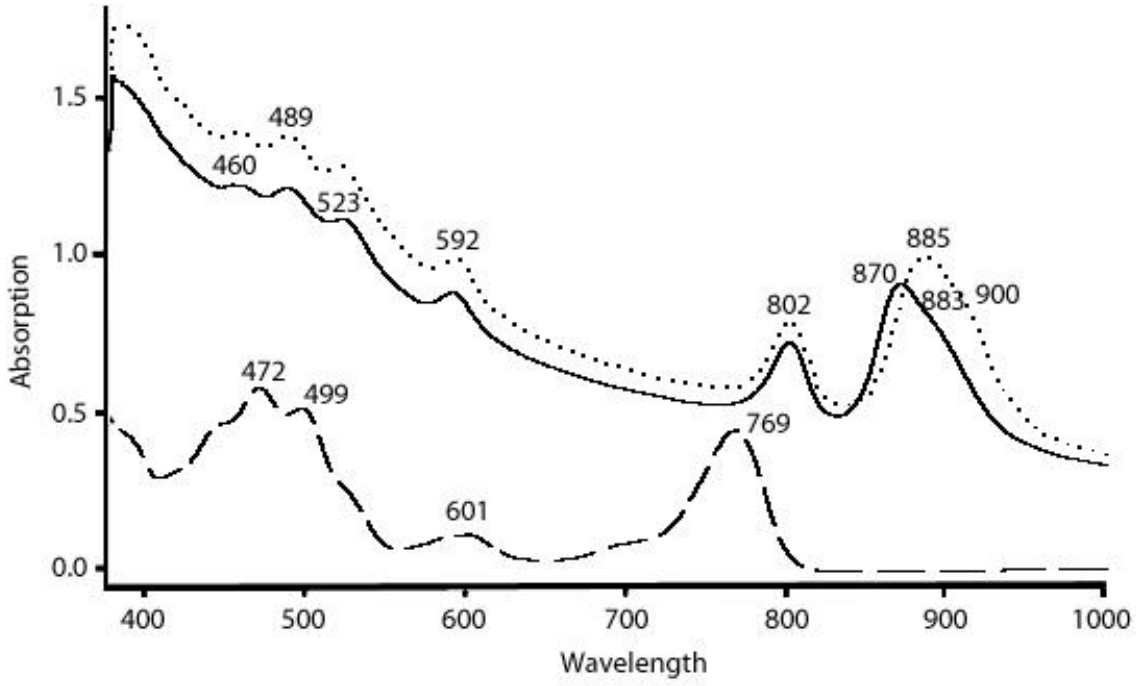

Fig. 11. Differences in the photosynthetic apparatus organization revealed from *in vivo* absorption spectra of strains BF1 (solid line) and BF14 (dotted line) grown at the same illuminated anaerobic conditions. Dashed line shows the spectrum of BF14 from an acetone-methanol extraction.

3.2.5 Biochemical and Physiological Data

Strains were tested for utilization of 28 single carbon sources in the light under anoxic conditions. As observed in *R. vannielii* (Imhoff, 2005b), each of the three strains used acetate, butyrate, glutamate, malate, peptone, propionate, pyruvate, succinate and yeast extract (Table 7). BF1 and BF16 grew on aspartate, ethanol, fumarate and lactate, differing from *R. vannielii* which was able to use all but aspartate. BF1 was the only

strain to assimilate mannose and sucrose. While previously characterized *R. vannielii* strains have utilized butanol, formate, glycerol and methanol, none of the new strains exhibited growth on these substrates. There also was no consumption of benzoate, citrate, cysteine, fructose, glucose, glycolate, methionine, nicotinic acid or tartate. None of the new isolates hydrolyzed starch, gelatin, Tween 20 or Tween 60. Catalase activity was demonstrated as seen in the type strain (Imhoff, 2005b). Typical for the genus, dark anaerobic growth on pyruvate was not observed. No strains gasified or acidified glucose, fructose or sucrose. Weak photoautotrophic growth was found with sulfide and hydrogen, but not with sulfate, sulfite or thiosulfate. All strains grew from 7 ˚C to 37 ˚C. No growth occurred at 2 ˚C or 42 ˚C. Highest growth rate was at 29 ˚C in strains BF1 and BF16, while BF14 grew approximately 25% better at 33 ˚C demonstrating an adaptation to its site of isolation that is consistently warmer than locations outside of the cave. All strains were mildly acidophilic, at pH 6.0-6.5 resulting in highest cell yields. The pH tolerance ranged from 5.0 to 8.0, similar to *R. vannielii*. There were no salt or growth factor requirements and NaCl was tolerated at 1.0% by BF1 and BF14. No growth was observed at 1.0% NaCl by BF16 or at 2.0% in any strains. Sulfide was tolerated up to 4.5 mM by all strains except BF1, which withstood up to 5 mM, or 2 mM greater than published for *R. vannielii* sulfide tolerance (Imhoff, 2005b). Each strain demonstrated nitrogen fixation and none exhibited nitrate reduction. Dinitrogen, ammonium, glutamine, nitrate and yeast extract all served as nitrogen sources for BF1. BF14 used dinitrogen, yeast extract and glutamine for nitrogen, while BF16 could also utilize nitrate. *R. vannielii* is known to use ammonia, dinitrogen, Casamino acids and yeast extract as nitrogen sources and some strains grow poorly with nitrate and urea

(Imhoff, 2005b). BF1 showed susceptibility to penicillin, polymyxin B, chloramphenicol, streptomycin, ampicillin, nalidixic acid and kanamycin. BF14 was sensitive to chloramphenicol and streptomycin and resistant to polymyxin B, ampicillin and nalidixic acid. BF16 was resistant to penicillin and ampicillin and susceptible to polymyxin B, chloramphenicol, streptomycin, nalidixic acid and kanamycin.

Based on 16S rRNA sequencing, strains BF1, BF14 and BF16 belonged to the *Alphaproteobacteria* within *Hyphomicrobiaceae* showing 99.6%, 98.6% and 99.6% similarity to *R. vannielii*, respectively. The phylogenetic tree (Fig. 12) displays clustering of the novel strains within *Rhizobiales* indicating *Pedomicrobium australicum*, a freshwater budding non-phototroph, as the next closest relative.

The DNA G+C was determined to be: in BF1, 61.8 mol%; BF14, 62.7 mol% and BF16, 63.5 mol%. The range of G+C content in previously published *Rhodomicrobium* strains is 61.8-63.8 mol% (Imhoff, 2005b).

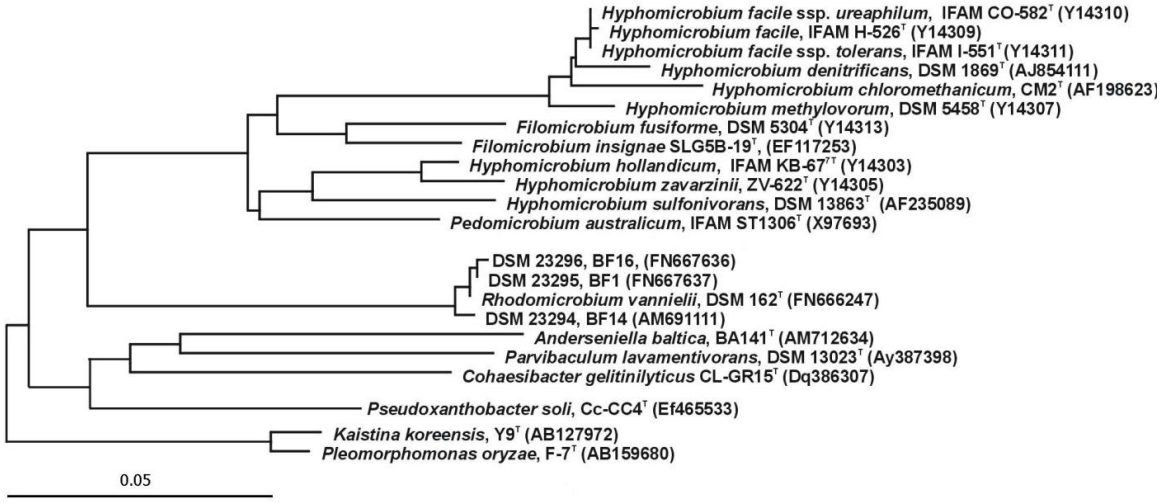

Fig. 12. Neighbor-joining dendrogram of 16S rRNA gene relatedness showing the phylogenetic positions of strains BF1, BF14 and BF 16.

Table 7. Comparative physiological characteristics of new isolates BF1, BF14, BF16 and type species *R. vannielii*.

Phenotype	*R. vannielii*	BF1	BF14	BF16
Cell width x length (µm)	1.2 x 2.8	0.8-1.9 x 1.1-3.2	1.0-2.3 x 0.9-1.3	0.9-1.4 x 1.2-2.8
16S rDNA similarity (%)	type strain	99.6	98.6	99.6
G+C content (mol%)	61.8-63.8	61.8	62.7	63.5
Bchl *a* Peaks (nm)	800-7, 869-72	802, 870	802, 885	802, 870
Carotenoid Peaks (nm)	461, 489, 523	61, 489, 524	461, 487, 521	461, 489, 521
Utilization of:				
Acetate	+	+	+	+
Aspartate	-	+/-	-	+/-
Benzoate	-	-	-	-
Butanol	+	-	-	-
Butyrate	+	+	+	+
Citrate	-	-	-	-
Cysteine	nd	-	-	-
Ethanol	+	+	-	+
Formate	some strains	-	-	-
Fructose	-	-	-	-
Fumarate	+	+	-	+
Glucose	-	-	-	-
Glutamate	-	+	+	+
Glycerol	some strains	-	-	-
Glycolate	-	-	-	-
Lactate	+	+	-	+
Malate	+	+	+	+
Mannose	-	+	-	-
Methanol	some strains	-	-	-
Methionine	nd	-	-	-
Peptone	nd	+/-	+/-	+
Propionate	+	+	+	+
Pyruvate	+	+	+	+
Succinate	+	+	+	+
Sucrose	nd	+	-	-
Tartate	-	-	-	-
Yeast extract	+	+	+	+
Hydrolysis				
Starch	nd	-	-	-
Gelatin	-	-	-	-
Tween 20	nd	-	-	-
Tween 60	nd	-	-	-
Catalase	+	-	-	-
Oxidase	nd	-	-	-
Fermentation	-	-	-	-
Autotrophy with:				
Sulfide	+	+	+	+
Thiosulfate	-	-	-	-
Sulfite	-	-	-	-
Sulfate	-	-	-	-
Hydrogen	+	+	+	+
Tolerances:				
Temp. range (^{o}C)	nd	7-37	7-37	7-37
Temp. optimum (^{o}C)	30	29	33	29
pH range	5.2-7.5	5.0-8.0	5.0-8.0	5.0-8.0
pH optimum	6.0	6.0-6.5	6.0-6.5	6.0-6.5
NaCl	nd	up to 1%	up to 1%	<1%
Sulfide (mM)	3.5	5.0	4.5	4.5
NaCl requirement	-	-	-	-
Nitrogen fixation	+	+	+	+
Nitrate reduction	nd	-	-	-
Resistance to:				
Penicillin	nd	-	nd	+
Polymyxin B	nd	-	+	-
Chloramphenicol	nd	-	-	-
Streptomycin	nd	-	-	-
Ampicillin	nd	-	+	+
Nalidixic acid	nd	-	+	-
Kanamycin	nd	-	nd	-
Growth factors	-	-	-	-

+, Growth or resistant; -, no growth or sensitive; +/-, weak growth; nd, not determined

3.2.6 Description of R. vangemerdenii

Of the three strains investigated, BF1and BF16 were the closest phenotypically to each other and to *R. vannielii*. Differences in ability to utilize sucrose and mannose, preference for nitrogen sources, tolerance to salinity and sulfide and antibiotic resistance profiles as well as DNA G+C content supported their sufficient dissimilarity to each other and type species *R. vannielii*. Together with genomic data, key traits including photosynthetic pigments and the photosynthetic apparatus, replication by budding, production of prosthecae and exospores, range of carbon sources and electron donors clearly related these isolates to *R. vannielii* and were thus presented as novel strains of this species.

Sequence dissimilarity of the 16S rRNA gene and significant phenotypic differences between BF14 and *R. vannielii* contributed to the separate classification within the genus *Rhodomicrobium* for which the species name *R. vangemerdenii* is proposed. These differences included distinction in at least five useable carbon sources (butanol, ethanol, fumarate, glutamate and lactate), a sulfide tolerance at least 1.0 mM higher than previously reported, obvious culture aggregative tendencies, an elevated temperature optimum and most notably the disparate spectral features including new two types of LH complexes previously unseen in *R. vannielii*.

Rhodomicrobium vangemerdenii (van. ge.′ mer. den.i.i., from van Gemerden, personal name. M. L. obj. *vangemerdenii*, of van Gemerden, to honor Hans van Gemerden for his contributions to studies of anoxygenic photosynthesis).

Individual cells are Gram negative, almond to ovoid shaped, 0.9-1.3 μm wide and 1.0-2.3 μm long producing prosthecae that terminate in spherical buds which develop into motile swarmer cells or non-motile exospores. Exospores measure 0.8-1.3 μm in diameter. Colony pigmentation is orange brown to red-purple under anoxic conditions and colorless to bright orange aerobically. Good anaerobic photoheterotrophic growth is exhibited using malate, succinate, pyruvate and yeast extract and weakly with glutamate and peptone as carbon sources; weak chemoorganotrophy occurs in the presence of yeast extract and anaerobic photolithoautotrophic growth is achievable with H_2S and H_2 as electron sources. Aerobic dark chemolithoautotrophy (with H_2) and fermentation of sugars are not observed. Starch, gelatin, Tween 20 and Tween 60 are not hydrolyzed. Optimum temperature for growth is 33 ˚C, with growth occurring as low as 7 ˚C and as high as 37 ˚C. Sulfide is tolerated up to 4.5 mM. No NaCl or growth factors are required. Glucose, fructose and lactose are not acidified. Resistant to ampicillin, nalidixic acid and polymyxin B and sensitive to chloramphenicol and streptomycin. Nitrogen can be fixed, nitrate reduction is not observed and glutamine and yeast extract are preferred as N-sources over N_2. Ammonium and nitrate are not suitable N-sources. The *in vivo* absorption spectrum of intact cells reflects the production of Bchl *a* in major peaks at 803 and 885 nm and carotenoids of the spheroidene series with peaks near 457, 488 and 522. A LH complex shoulder is observed at 897-900 nm. The DNA G+C content is 62.7 mol% and 16S rRNA gene sequencing reflects a 98.6% similarity to *Rhodomicrobium vannielii*. The type strain of the species is $BF14^T$ (=DSM 23294).

The habitat of BF14^T is a submerged sulfidic green-sulfur bacteria-dominated mat located at the Upper Middle Springs on Sulphur Mountain in Banff National Park, Alberta, Canada.

3.3 *Rhodobacter fonsus* sp. nov., a purple nonsulfur Alphaproteobacterium isolated from Sulphur Mountain Thermal Springs in Banff, Alberta, Canada

3.3.1 Abstract and Opening Remarks

A facultative anaerobic strain BF9^T was isolated from a cyanobacteria-dominated submerged mat in the Upper Cave and Basin spring pool in Banff, Alberta. BF9^T, a Gram negative ovoid to rod-shaped purple nonsulfur bacterium, formed long chains and grew well under photoheterotrophic and chemoorganotrophic conditions. Autotrophic growth was possible with CO_2 and sulfide or hydrogen as electron donors. No growth factors were required. Spheroidene and spheroidenone were the major cell carotenoids. 16S rRNA gene sequence analysis indicated moderate relatedness to *Rhodobacter capsulatus* ATCC 11166^T (98.0% sequence similarity). The DNA G+C content was 71.8 mol%. Phylogenetic position and key phenotypic dissimilarity distinguished it as a novel species of the genus *Rhodobacter* for which the name *R. fonsus* is proposed. The type strain is BF9^T (=DSM 23281^T, =VKM B-2661). The accession number of the 16S rRNA gene sequence of BF9^T is AM691096.

From Himalayan snows (Arunasri *et al.*, 2008; Kumar *et al.*, 2007) to the Adriatic Sea (Imhoff *et al.*, 1984) and from the sediments of polluted ponds (Srinivas *et al.*, 2008)

to Antarctic Lakes (Karr *et al.*, 2003), *Rhodobacter* strains have been isolated from disparate habitats globally. The 12 species of the *Rhodobacteraceae* type genus (Euzeby, 1997) are metabolically diverse purple nonsulfur photoheterotrophs sharing the presence of LHI (absorbance peaks range from 870 to 890 nm) and LHII (absorbing near 800 nm and at around 820-850nm depending on species) (Cogdell *et al.*, 2004; Imhoff, 2005c), orange-brown to green-brown pigmentation of anaerobic cultures, carotenoids of the spheroidene series, vitamin requirements and a large-type soluble periplasmic cytochrome c_2 and Q-10 quinone component (Imhoff, 2005c; Ramana *et al.*, 2009). Before the discovery of halophilic *Rba.vinaykumarii* (Srinivas *et al.*, 2007), all *Rhodobacter* species were distinguished from close relatives in the genus *Rhodovulum* by lack of NaCl requirement (Imhoff, 2005c). Distinction from phylogenetically close purple nonsulfur *Rhodobaca* included the presence of both LH complexes and from *Rubrivivax* by a number of chemotaxonomic traits such as lack of c_2 and Q-10 (Imhoff, 2005c). Further distinction from other genera and phylogenetic positioning has since rested in sequencing of 16S rRNA genes (Ramana *et al.*, 2008; Ramana *et al.*, 2009) though two significant reclassifications of similar organisms has gone beyond subunit sequencing. Represented by a single clinical isolate until 2007, *Rhodobacter massiliensis* (Greub & Raoult, 2003) was reclassified to represent a new genus, *Haemotobacter*, following the polyphasic analysis of a dozen of its 16S rRNA gene sequence relatives obtained on blood cultures (Helsel *et al.*, 2007). In addition to their human reservoirs- all other *Rhodobacter* species having been isolated from aquatic environments- key discriminating characters included disparate cellular fatty acid profiles, ≥2.6% lower G+C mol% content, obligate aerobic metabolism and lack of genes coding for a

photosynthetic apparatus (Helsel *et al.*, 2007). Absence of *puh*A and *puf* genes was also one of the foremost distinctions preventing non-phototrophic *Pseudorhodobacter* (formerly *Agrobacterium*) *ferrrugineum* from classification within the *Rhodobacter* genus, though it remains branched within the clade (Uchino *et al.*, 1997; Uchino *et al.*, 2002).

3.3.2 Taxonomic Characterization Test Results

Young plate cultures of BF9^T (<48 hours) were of ovoid morphology exhibiting multiplication by binary fission (Fig. 1A) as found in all *Rhodobacter* species excluding *R. blasticus* (Imhoff, 2005c). Cell width was 0.5-1.3 µm and length, 1.3-3.0 µm. Older cultures exhibited characteristic *Rhodobacter* straight chain formation of typically 4-8 cells (Fig. 1B), but often up to one dozen measuring 25 to 40 µm in length. Aerobic cultures began colorless developing a pink-purple pigmentation within 48 hours, while anaerobic agar deep cultures were olive green to yellow brown. Capsule production, a trait that varies between strains of *R. capsulatus* (Ramana *et al.*, 2009), was not observed.

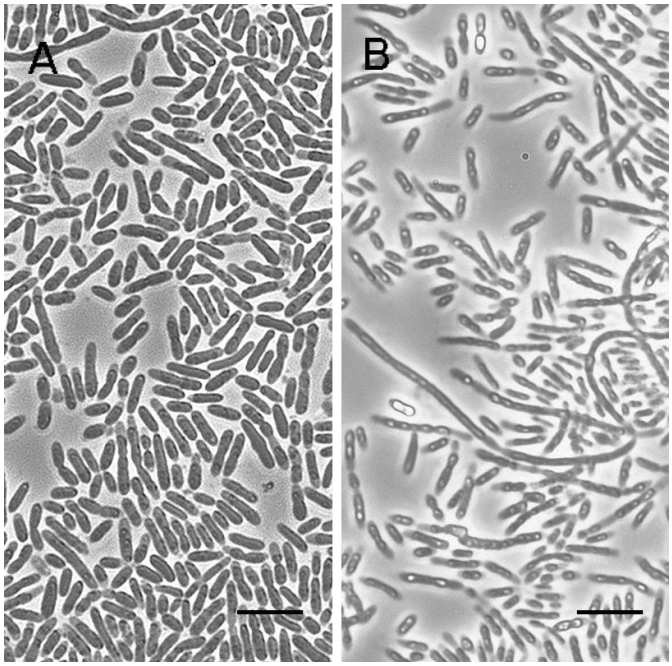

Figure 13. Phase contrast micrographs of BF9 grown chemoheterotrophically: (A) ovoid to rod-shaped cells from a plate culture after 48 hours of growth, (B) chain formation and intracellular storage products. Bar, 5 µm.

Taxonomic test results are displayed in Table 8, featuring discriminating characters of BF9^T to its relatives *R. capsulatus*, *R. aeustuarii* and *R. maris*. Photoheterotrophic growth was demonstrated as well as chemoheterotrophy (on pyruvate, 0.2%; succinate, 0.2% and yeast extract, 0.05%) and photolithoautotrophy (with H_2, 20% and NaH_2S, 0.5 mM). Photoautotrophic growth with H_2 as an electron donor was comparatively slow, a feature also observed in *R. sphaeroides* (Imhoff, 2005c). In contrast to *R. capsulatus*, chemolithoautotrophy using H_2 and fermentation of pyruvate and succinate (at 0.3% each) were not observed. Substrates used by BF9^T for photoorganotrophic growth

Table 8. Differential characteristics of strain BF9^T and closely related *Rhodobacter* species.

Phenotype	BF9^T	*R. aestuarii**	*R. capsulatus**	*R. maris**
Cell size (microns)	0.5-1.3 x 1.3-3.0	0.7-1.0 x 1.5-2.0	0.5-1.2 x 2.0-2.5	0.6-1.0 x 1.0-1.5
G+C content (%)	71.8	65.1	68.1-69.6	62.9
Capsule production	-	-	-/+	-
Utilization of:				
acetate	+	-	+	(+)
aspartate	-	-	-/+	-
citrate	-	-	-/+	-
ethanol	-	-	-	-
formate	-	-	+	-
fructose	+	-	+	-
fumarate	+	-	+	(+)
D-glucose	+	-	+	-
glutamate	+	-	+	-
glycerol	-	-	-	(+)
lactate	+	(+)	+	(+)
malate	+	-	+	+
methanol	-	nr	-	nr
propionate	+	-	+	(+)
tartate	-	-	-	-
Autotrophy with:				
sulfide	(+)	-	+	-
thiosulfate	-	-	-	-
hydrogen	(+)	-	+	nr
Temperature optimum (°C)	29-34	25-30	30-35	25-30
pH range	6.5-8.5	6.0-8.5	6.5-7.5	5.0-8.0
pH optimum	7.0-7.5	7.0	7.0	6.5-7.0
NaCl requirement	-	-	-	-
Nitrate reduction	-	-	-/+	-
Growth factor required	-	t	t (b, n)	t

+, positive; -, negative; -/+, variable by strain; (+), weak; nr, not reported; t, thiamine; b, biotin; n, niacin

included: acetate, butanol (weakly), casamino acids, fructose, fumarate, glucose, glutamate, lactate, malate, peptone, propionate, pyruvate, succinate, sucrose and yeast extract. Substrates tested that could not be utilized were aspartate, benzoate, citrate, cysteine, ethanol, formate, gluconate, glycerol, methanol, methionine and tartate. These results differ in the use of formate and citrate, the former used by *R. capsulatus* and the latter used variably by different strains (Ramana *et al.*, 2009). Tween 20, Tween 60 and

starch were not hydrolyzed, though gelatin hydrolysis was possible. NaCl was not required for growth of BF9^{T}, but was tolerated up to 2.0%. Nitrate reduction, a variable trait in *R. capsulatus* strains, was not detected. Optimal growth was observed at 29-34 ˚C within a range of 10 to 37˚C and at pH 7.0-7.5 within the range 6.5 to 8.5; all typical of the genus. No vitamins were required as growth factors, a key difference with other members of *Rhodobacter*, though OD was observed to be roughly one-fifth less in the thiamine-lacking medium compared to that of the full vitamin-containing medium indicating some stimulation of growth by thiamine. Molecular nitrogen, ammonium chloride, glutamine and yeast extract all served as suitable nitrogen sources and magnesium sulfate, thiosulfate, sulfite and methionine were utilized for sulfur. Sulfide alone was not suitable to provide sulfur for biosynthesis, although was tolerated up to 4.0 mM. The *in vivo* absorption spectrum of photoheterotrophic cells matched that of typical *Rhodobacter* profiles (Imhoff, 2005c; Kumar *et al.*, 2007) exhibiting maxima at 446-51, 475-79 and 510 nm, confirming the presence of carotenoids, as well as at 590, 802 and 860 with a shoulder near 874 nm, indicative of Bchl *a* (Fig. 14).

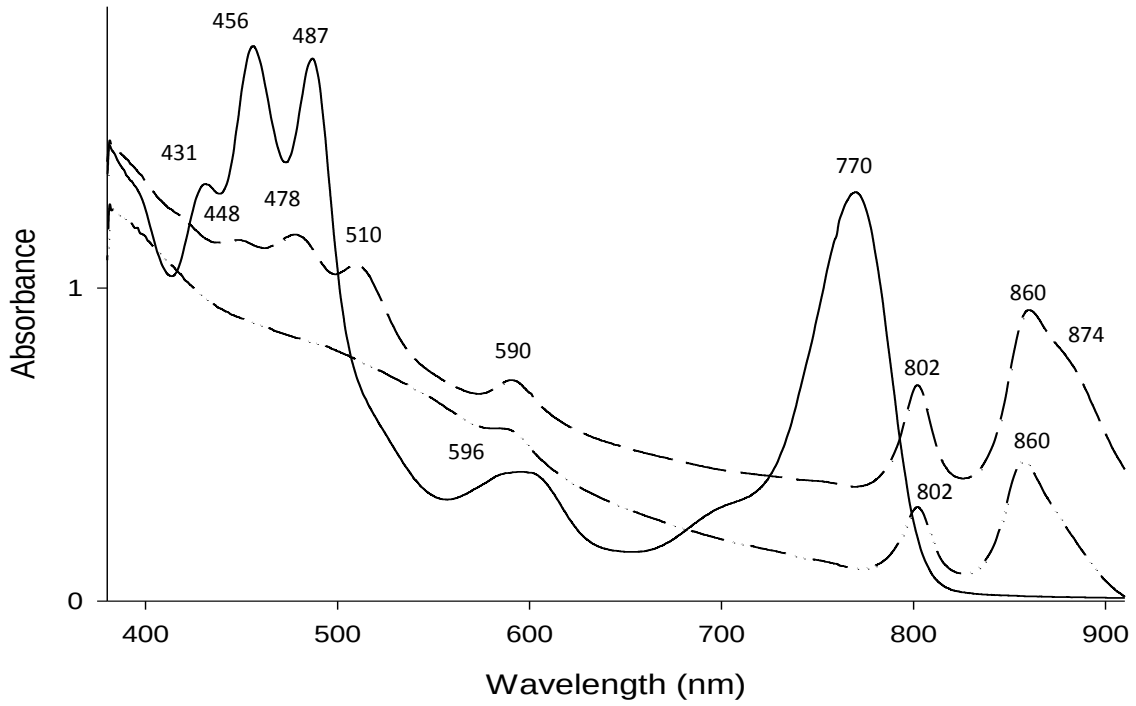

Figure 14. Absorption spectra of anaerobic photoheterotrophic BF9 culture by acetone:methanol extraction (solid line), *in vivo* with 10 mM Tris buffer and bovine serum albumin (dashed line) and *in vivo* whole cell aerobic, dark incubated culture with 10 mM Tris and BSA (dot-dash line).

In contrast, the absorption spectrum of aerobic cultures lack the distinct carotenoid peaks and the LHI shoulder observed under anaerobic conditions. Pigments extracted in acetone:methanol (7:2) yielded absorption maxima at 431, 456 and 487 nm, indicating spheroidene and spheroidenone as the major carotenoids, and maxima at 381, 595 and 770 nm confirming the presence of Bchl *a* (Fig. 14).

Fig. 15 exhibits phylogenetic positioning of BF9^T relative to members of *Rhodobacter* and other purple nonsulfur bacteria. 16S rRNA gene sequence analysis revealed that strain BF9^T showed 98.0% similarity to *R. capsulatus* ATCC 11166^T. HPLC analysis of

DNA determined the G+C content to be 71.8 mol%, which is significantly higher than that of *R. capsulatus* ATCC 11166^T (65.5-66.8 mol%) (Ramana *et al.*, 2009).

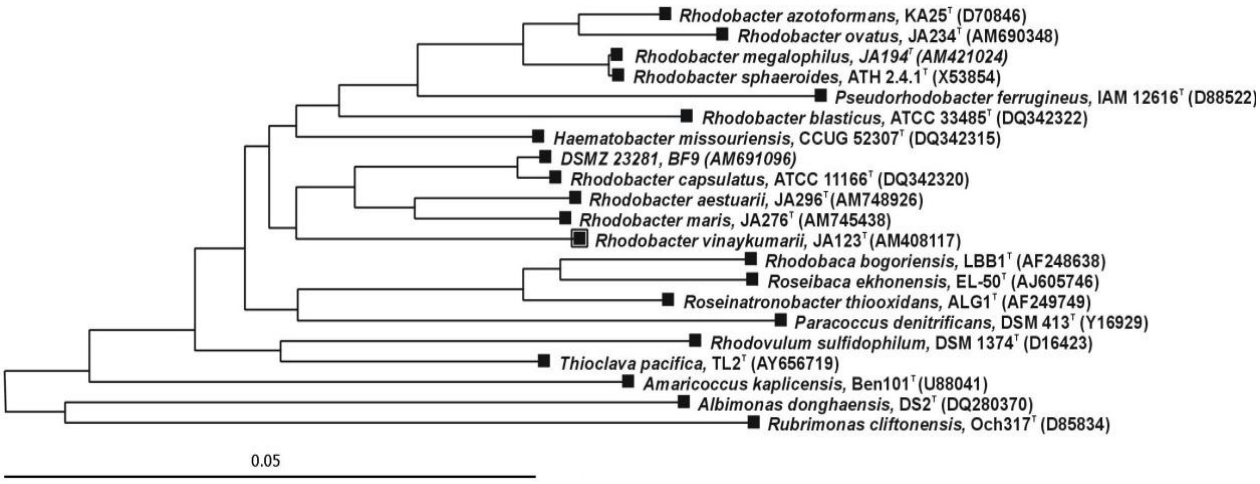

Figure 15. Evolutionary-distance dendrogram depicting the phylogenetic relationships of strain BF9^T within *Rhodobacteraceae*, determined using 16S rRNA gene sequence analysis. Bootstrap values support branching at higher than 50% confidence. Black dots represent identical branch points with those found using the DNAPARS algorithm with Felsenstein correction.

3.3.3 Description of R. fonsus

The 16S sequence and G+C content disparity, together with phenotypic differences (long chain-formation, inability to use formate, increased tolerance of alkalinity and distinguished lack of vitamin requirement; see Table 8) warranted the description of BF9^T as a new species of *Rhodobacter* for which the name *Rhodobacter fonsus* was proposed.

Rhodobacter fonsus (fon.′sus. M. L. m. n. *fons* spring [water], reflecting the location of isolation).

Cells are motile, ovoid to pleomorphic rods, 0.5-1.2 µm wide and 1.3-3.0 µm long that form chains from 3 to 12 cells long. Capsule is not formed. Culture color is pink-purple aerobically and olive green-brown under anoxic conditions. Anaerobic photoheterotrophic, anaerobic photolithoautotrophic and aerobic chemoorganotrophic modes of growth are all possible. Chemoautotrophic growth and fermentation have not been demonstrated. Bchl *a*, spheroidene and spheroidenone are the major photosynthetic pigments with i*n vivo* spectral maxima occurring at 448, 478, 510, 590, 802, 860 and 874 nm. Neither vitamins nor salt are required, though presence of thiamine stimulates growth and NaCl can be tolerated up to 2.0%. Sulfide can be tolerated up to 4.0 mM. Gelatin hydrolysis, catalase and oxidase tests are positive and starch hydrolysis is negative. Growth occurs at pH 6.5 to 8.5 (optimum 7.0-7.5) and 10 to 37 ˚C (optimally 29-34 ˚C). The DNA G+C content is 71.8 mol%. The type strain is BF9^{T} (=DSM 23281^{T}).

The habitat of BF9 is a purple sulfur bacteria-dominated submerged mat from a sulfidic mesothermal (28-36 °C) spring on Sulphur Mountain in Banff National Park, Alberta, Canada.

3.4 *Erythromicrobium magnum* sp. nov., an aerobic anoxygenic phototroph isolated from Sulphur Mountain Thermal Springs in Banff, Alberta, Canada

3.4.1 Abstract and Opening Remarks

A motile, orange-red, short rod strain BF8 was isolated from a floating cyanobacteria mat on an outflow pool at the Sulphur Mountain thermal springs of Banff National Park, Alberta, Canada. BF8 contained Bchl *a* incorporated into RC and LHI complexes absorbing maximally at 806-808 nm and 865-867 nm. 16S rRNA sequencing revealed 98.9% similarity to *Erythromicrobium ramosum*. The LHII complex absorbing near 832 nm, characteristic of *Erythromicrobium* species, was not present. Cultures of BF8 preferentially grew chemoheterotrophically, could not grow under anoxic conditions and produced Bchl *a* only in the dark, which is typical of aerobic anoxygenic phototrophic bacteria. The DNA G+C content was 67.1 mol%. The spectral properties and disparate physiological and phylogenetic traits support the proposal of the novel species *Erythromicrobium magnum* sp. nov. with BF8^T (=DSM 23304^T) as the type strain.

Strain BF8 was isolated from Site 3, the *Phormidium*- and *Spirulina*-dominated floating mat at the C&B outflow pool in May 2005. The type strain E5 for *Erythromicrobium ramosum*, the closest phylogenetic relative of BF8, was also isolated from a mesothermal (25 ˚C) cyanobacterial mat, but one of alkaline pH 9.5 (Yurkov *et al.*, 1994).

3.4.2 Taxonomic Characterization Test Results

On agar plates BF8 formed shiny deep orange-red convex colonies up to 3 mm diameter. Lawns of growth on RO agar plates became dry and difficult to scrape from the agar

surface within 3 days of incubation contrary to *E. ramosum*, E5 mucoid texture lasting at least up to a week at 28 ˚C. In liquid culture BF8 aggregated into clumps several millimeters in diameter though E5 developed as a turbid, non-aggregating suspension.

Growth did not occur anaerobically in the light or the dark in PNS medium. After demonstrating BF8 was a strictly aerobic facultative photoheterotroph that produced Bchl *a* only in the dark, the designation of the strain as an aerobic anoxygenic phototrophic bacterium logically followed (Yurkov & Csotonyi, 2009).

Cells grown exponentially were motile, coccoid-ovoid to short rods 0.6-1.1 µm wide by 1.0-1.9 µm long. Division occurred by binary and multiple fission or by asymmetric constriction. Pleomorphism was observed in some rods having bulging ends and ovoid cells with tapered ends (almost lemon-shaped) and included branching similar to that reported for *E. ramosum* (Yurkov *et al.*, 1994) (Fig. 16C and D).

Log phase BF8 cultures yielded absorption value ratios of Bchl *a* to carotenoids near 1:3. This is an unusually large proportion compared to the approximate 1:7 ratio of Bchl *a* to carotenoids observed in E5 (Yurkov & Beatty, 1998). Major *in vivo* absorption spectral peaks occurred at 457 and 483 nm and acetone:methanol-extracted (7:2) profiles reared maxima at 460-63 nm and 475-477 nm, suggesting presence of the carotenoids erythroxanthin sulfate and bacteriorubixanthinal, also found in E5 (Yurkov *et al.*, 1993). *In* vivo Bchl *a* peaks were present at 806-808 and 865-867 nm indicating a photosynthetic apparatus organized into LHI and RC. The distinguishing LHII peak of *E. ramosum* at 830-832 nm (Fig. 17) was absent in BF8.

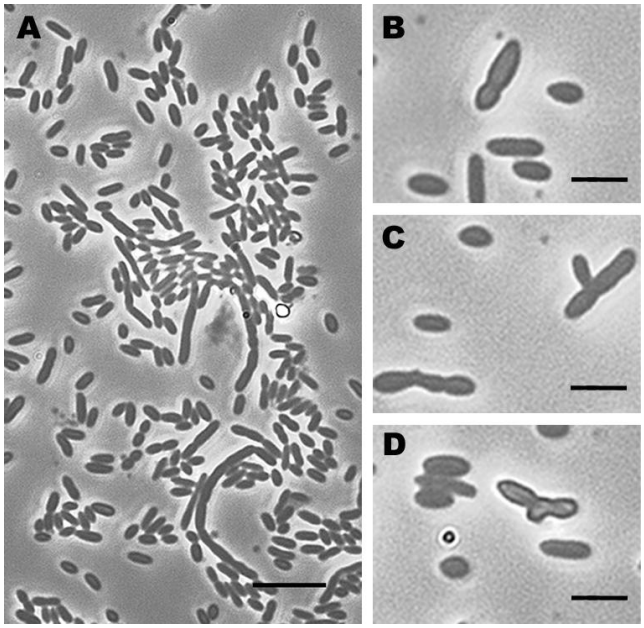

Fig. 16. Phase-contrast micrographs of exponential phase BF8 cultured on RO agar plates. (A) displays general ovoid to rod morphology (Bar, 5 µm), (B) shows asymmetrical constriction form of reproduction, (C) captures multiple fission and branching, (D) branching as in *E. ramosum*. B-D Bar, 2 µm.

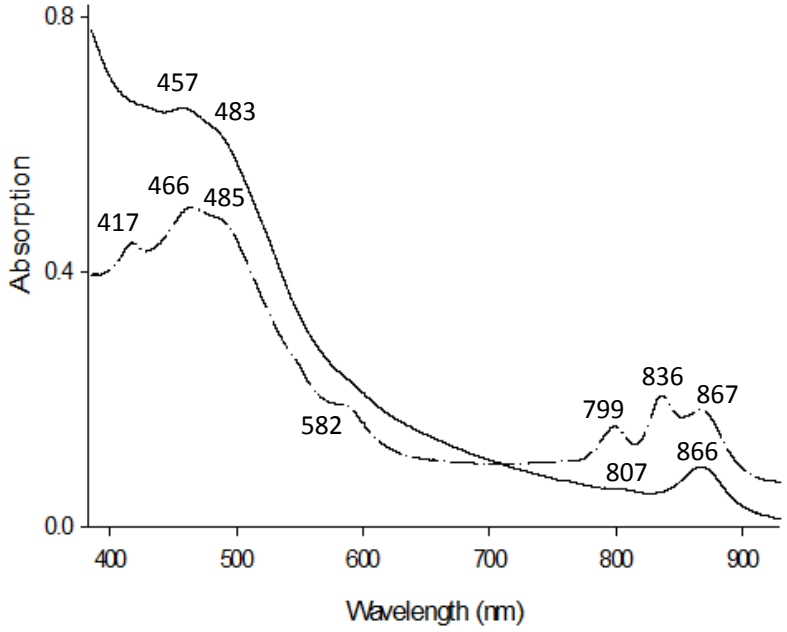

Fig. 17. *In vivo* spectral profiles of strain BF8 (dashed line) and *Erythromicrobium ramosum* type strain E5 (solid line) cultured in the dark on RO agar plates.

The new isolate exhibited aerobic chemoheterotrophic utilization of acetate, butanol, butyrate, casamino acids, citrate, cysteine, dextrose, fructose, glutamate, lactate, maltose, peptone, propionate, pyruvate, sucrose, xylose and yeast extract, but was unable to use aspartate, benzoate, ethanol, formate, gluconate, glycerol, malate, methanol, nicotinic acid, phenylalanine, proline, ribose, succinate and tartate. Contrary to BF8, *E. ramosum* was shown to assimilate ethanol, malate proline and succinate (Yurkov, 2005). Anaerobic photoheterotrophy, chemoautotrophy, photoautotrophy or fermentative growth were not observed. Glucose, lactose and sucrose were all acidified, but no CO_2 was produced. The tests for catalase, gelatin hydrolysis and amylase were positive, differing from *E. ramosum* in its lack of gelatinase and ability to hydrolyze starch. Oxidase activity, present in *E. ramosum*, was absent in BF8 as was hydrolysis of Tween 60. Growth occurred from pH 6.0 to 9.0 with optimal range at 7.0-7.5 and at a temperature range of 7 to 37 ˚C, with optimum at 30-34 ˚C. NaCl was not required, but tolerated up to 4.0% and while no vitamins were necessary as growth factors, the presence of thiamine increased biomass. As found for *E. ramosum*, nitrate reduction was not demonstrated. Uninhibited growth occurred in the presence of antibiotics penicillin, streptomycin, tetracycline, ampicillin and nalidixic acid, however sensitivity to polymyxin B, chloramphenicol and kanamycin was observed. *E. ramosum* was found to have the same responses to these antibiotics, except in its sensitivity to tetracycline and nalidixic acid (Yurkov, 2005).

Sequence analysis of 16S rRNA showed 98.9% similarity to *E. ramosum*, E5 and DNA G+C content was determined to be 67.1 mol%. The DNA G+C content of E5 was found to be lower than BF8, comprising only 64.2 mol% (Yurkov, 2005). Fig. 18

displays the neighbor-joining dendrogram indicating phylogenetic relatedness of BF8 to other *Sphingomonadaceae*. It is most closely related to *Erythromicrobium ramosum* followed by *Porphyrobacter sanguineum*. The sequence accession number for BF8 is FN668376.

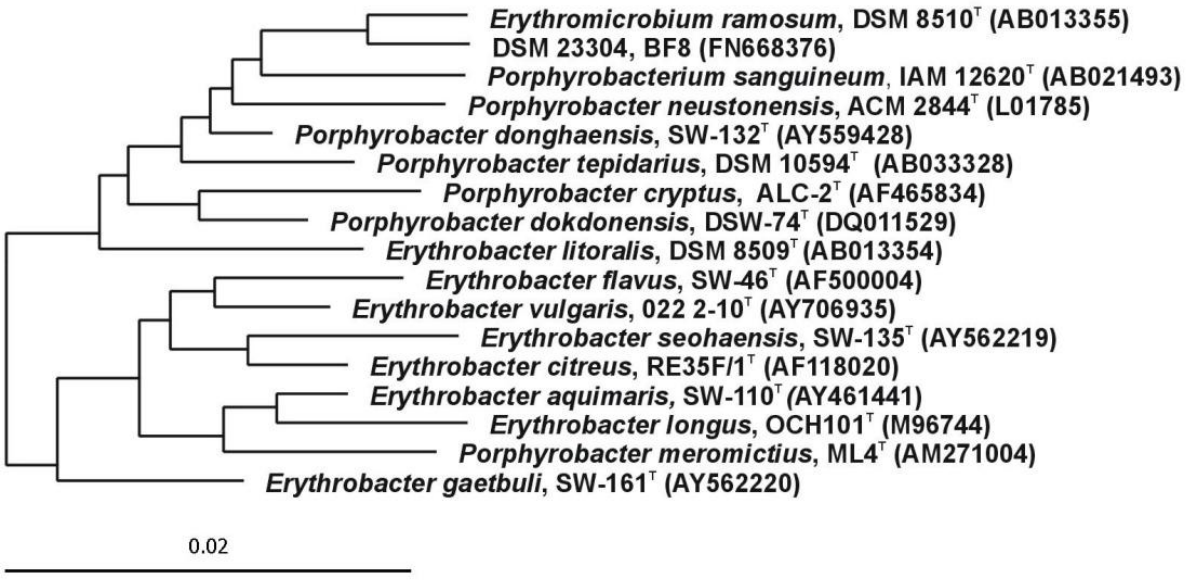

Fig. 18. Evolutionary-distance dendrogram depicting the phylogenetic relationships of strain BF8 within *Sphingomonadaceae*, determined using 16S rRNA gene sequence analysis.

3.4.3 Description of E. magnum

Differences between type strain *E. ramosum* and BF8 thus included utilization of ethanol, malate, proline, succinate and xylose; a higher temperature optimum for BF8 (30-34 ˚C compared to 25-30 ˚C), a lack of oxidase and its resistance to tetracycline and kanamycin. Together with genomic disparity and differences in the organization of the photosynthetic

apparatus, these phenotypic distinctions support the addition of BF8 as a new species to *Erythromicrobium* for which the name *E. magnus* was proposed.

E. magnum (mag.′num. L. masc. adj. m*agnum* great, reflecting both the organism's array of morphology and its abundance throughout the Sulphur Mountain spring system).

Cells are ovoid to short rods 0.6-1.1 µm x 1.0-1.9 µm in size. Produce Bchl *a* and carotenoid pigments. *In vivo* absorption spectra exhibit RC and LHI peaks at 806-808 nm and 865-867 nm. Carotenoid peaks show maxima at 460-463 nm and 475-477 nm in acetone:methanol (7:2) indicating erythroxanthin sulfate and bacteriorubixanthinal. The strain is an obligate aerobe and facultative photoheterotroph. Chemoheterotrophy can occur on acetate, butanol, butyrate, casamino acids, citrate, cysteine, dextrose, fructose, glutamate, lactate, maltose, peptone, propionate, pyruvate, sucrose, xylose and yeast extract. Aspartate, benzoate, ethanol, formate, gluconate, glycerol, malate, methanol, nicotinic acid, phenylalanine, proline, ribose, succinate and tartate are not used as a carbon source. Catalase, amylase and gelatinase tests are positive and oxidase activity is not observed. Optimal temperature for growth is 30-34 ˚C at a pH of 7.0-7.5. The species can grow within a range of 7 to 37 ˚C and pH 6.0 to 9.0. Neither growth factors nor NaCl are required, but salinity is tolerated up to 4.0% and thiamine stimulates growth. Growth occurs in the presence of penicillin, streptomycin, tetracycline, ampicillin and nalidixic acid, but not polymyxin B, chloramphenicol or kanamycin. The DNA G+C content is 67.1 mol%. The type strain is $BF8^T$ (=DSM 23304^T).

The habitat of $BF8^T$ is the mesothermal sulfidic springs of Sulphur Mountain in Banff National Park, Alberta, Canada.

4 Conclusions

4.1 Summary of Results

The foundation of this investigation of the Sulphur Mountain spring mat communities was laid by obtaining a census of anoxygenic phototroph populations in different seasons, a qualitative assessment of the dominant oxygenic phototrophs within the community, profiles of the major photosynthetic pigments and an estimate of their concentrations, rates of carbon fixation facilitated by the microbial mat, the beginnings of a prokaryotic species catalogue based upon morphotype, spectral properties and, for several isolates, 16S rRNA gene sequencing and a comparison of these microbial dynamics to fluctuations in the population of *P. johnsoni*.

PNSB were found to be the most populous of the three groups of anoxygenic phototrophs reaching densities near 4.8 x 10^5 CFU/cm^2 in summer 2003, the greatest numbers being associated with cyanobacteria-dominant sampling sites more than those of *Thiothrix*. AAP ranged from non-detection, <1 CFU/cm^2 in several seasons, to maxima of 2.3 x 10^5 CFU/cm^2 (Site 3, Aug. 2003) and 3.4 x 10^5 CFU/cm^2 (Site 3, Feb. 2005). True PSB isolates were limited to submerged sites, isolated in concert with sulfide-tolerant PNSB in greatest number during the late summer and autumn e.g. 5.2 x 10^5 CFU/cm^2 at Site 8 and 1.1 x 10^5 CFU/cm^2 at Site 9, each in October 2005.

As expected, the major photosynthetic pigments were confirmed to be Chl *a* and *b* from a consistent dominance of cyanobacterial strains resembling the genera *Spirulina*, *Oscillatoria*, *Phormidium* and *Synechocystis* and eukaryotic *Aphanothece*, *Dermocarpa*

and *Chlorococcum*. Significant contents of Bchl *a* were quantified in the fall and early winter and found to be greater at cyanobacterial sites than those dominated by *Thiothrix*.

Mat carbon fixation was measured to be extremely high with significant non-light driven autotrophy rates discovered in November and greatest total activity consistently found in spring (May 2005 and 2007), facilitated by oxygenic phototrophs. Anoxygenic phototrophic fixation was generally negligible (<5% of total fixation) but reached ~10-30% of total fixation in several seasons (e.g. March 2006, May 2005 and 2007) and once over 90% at Site 12 in October 2005.

Regarding bacterial diversity, three novel species and two strains have been characterized and submitted for publishing. In addition, other strains or potential species of *Rhodomicrobium* (BF13), *Paracraurococcus* (BF15), *Porphyrobacter* (BF6), non-phototrophic *Thermomonas* (BF21) and *Brevundimonas* (BF4) and several unsequenced orange AAP (BF61-68) have had full or extensive taxonomic data prepared. The Banff springs proved to be rife with unexplored diversity, though major PNSB community members, *Rhodobacter*, *Rhodopseudomonas* and *Rubrivivax*, were consistent with previous studies of similar mat communities (Mehrabi *et al.*, 2001; Overmann & Garcia-Pichel, 2006).

Relating microbial dynamics to *P. johnsoni* population changes was dependent upon discerning patterns visually rather than through statistical analysis due to lack of sufficient trials. Replicates obtained showed somewhat opposing trends in the abundance of grazers and the amount of prokaryotic nutritive resources available. A lag of several months appeared to separate the maximal seasonal development observed for the

microbial mat in late summer and the highest of snail numbers in early winter. Described in other ecological contexts as *tracking inertia* (Solbreck & Sillen-Tullberg, 1986), it is the time required for a natural population to respond to changes in the ecosystem. Natural populations face constant temporal and spatial variability and never quite establish any theoretical equilibrium proportional to the influences found within their niche. Response and adjustment to new inputs- positive or negative- are stifled or delayed by the myriad other environmental factors at play. The two major variables indicating organic inputs were mat expanse and mat-facilitated C_i-fixation. Positive trends co-occurring between prokaryotes and snails included mat Chl *a* content and the density of Bchl *a*-containing strains per unit area. Exact influence of either parameter on snail fecundity was not certain, but may be explained in terms of season (i.e. maximum Chl *a* per cell produced in months with lower light availability) and a G→P postulate (i.e. larger grazer input stimulates greater photoheterotrophic bacteria proliferation).

4.2 Future Prospects

While microbial ecology papers have increasingly moved towards relying solely on molecular techniques (Allen *et al.*, 2009; Dillon *et al.*, 2008; Goh *et al.*, 2009), classical culturing is the only means of obtaining pure isolates for phenotypic characterization. Ideally the two approaches would be used in concert: molecular techniques such as denaturing gradient gel electrophoresis (DGGE) may guide enrichment culturing and expand on the what can be learned from the small (~1%) percentage of viable or easily cultivated organisms isolated in the laboratory (Kimura, 2006). Morphology and absorption spectra characteristics are also known to conceal phylogenetic diversity when grouping organisms by eye. Future investigation of the Banff Springs microbial mats

would best include DGGE of PCR-amplified 5S rRNA subunits (Moyer *et al.*, 1995; Stahl *et al.*, 1985) or 16S rRNA gene segments (Ferris & Ward, 1997; Hongmei *et al.*, 2005; Lunina *et al.*, 2007) or even whole genome shotgun sequencing, the in-depth metagenomics approach taken, for example, by Venter *et al.* (2004) studying the microbial community of the Sargasso Sea. Highlights of the Venter *et al.* study included an unprecedented revelation of 148 novel bacterial phylotypes and over 1.2 million new genes derived from roughly 1800 genomic species. While the phylogenetic robustness of the whole-genome approach is presently unmatched, the sheer volume of labor involved in the analysis creates difficulty for any single graduate project to elucidate the temporal variation of a microbial community. An example of a model study may then be the Dillon *et al.* (2008) quantification of the spatial and temporal variability of the hypersaline benthic mats at Guerrero Negro, Baja CA Sur, Mexico. The study used a combination of timed sampling, terminal restriction fragment length polymorphism analysis, 16S rRNA gene sequencing and cryotome sectioning (at 50-µm intervals) of frozen core samples to determine seasonal, diel and horizontal and vertical characteristics of the mat community. Tracing the change in vertical stratification of the mat community over the diel cycle would be interesting to compare to measurements of grazing intensity of *P. johnsoni* over the day-night cycle. While our culturing accounted for the community profiles of 1-cm^2 mat samples at thicknesses ranging from 1 mm to >1 cm, the radulas of *P. johnsoni*, as suggested by snails of slightly larger shell length (Bromley & Hanken, 1980) with teeth (in the example of *Physella gyrina*) measuring only 11-13 µm (Barnese *et al.*, 1990), feasibly reaches maximal grazing depths of around 100 µm. The stratification of the microbial community is thus quite pertinent and depending on

what time of day *P. johnsoni* grazes most, the diurnal vertical migrations of some motile cyanobacteria e.g. *Oscillatoria* (Richardson & Castenholz, 1987a) will be important to follow. Unfortunately, due to the distance of our facility to Banff sampling sites, lack of microbiological resources available at the Banff National Park Wildlife lab and funding, such approaches were not feasible for this stage of the project.

Controlled feeding experiments would also be of great interest. A future study may include pure or near-pure cultures of *Spirulina*, *Oscillatoria* and *Thiothrix* and detritus as sole nutrition sources and combined food choices. By limiting available food, the nutritional quality of the dominant mat organisms/organics can be observed through growth and mortality rates, as can *P. johnsoni*'s ability to choose foods that satisfy its somatic or reproductive needs as has been shown, for example, in *Limicolaria flammea* (Egonmwan, 1991).

Taking a step back, population dynamics of the prokaryotes and eukaryotes present within the Sulphur Mountain Thermal Springs system is inherently directed by the springs themselves. Previous spring drying and excessive precipitation events have had tremendous impacts on both *P. johnsoni* and the microbial mat. It may also be inferred that a temperature barrier separated the snail from colonization of the West Cave, Site 13. Hydrodynamics may then be the most crucial facet of the ecosystem to elucidate as well as the human response required to aid in the stabilization of faunal populations during such watershed geological and climate events.

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