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Symbiotické mikroorganismy a antimikrobiální aktivita *Pectinatella magnifica*

Disertační práce

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Prohlášení

Prohlašuji, že jsem disertační práci na téma: „Symbiotické mikroorganismy a antimikrobiální aktivita *Pectinatella magnifica*" vypracovala samostatně a použila pouze pramenů, které cituji a uvádím v přiložené bibliografii.

V Praze, dne 2. 8. 2018

Podpis autora práce: ....

Poděkování

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1. Úvod

Pectinatella magnifica (Leidy, 1851) je sladkovodní, bezobratlý živočich, který se řadí do kmene Bryozoa (Mechovky). Tvoří velké kolonie z gelového materiálu na povrchu ponořených substrátů, ve všech typech vodních toků. Ačkoliv se původně jedná o severoamerický druh, jeho výskyt byl záhy zaznamenán na mnoha lokalitách v Evropě, Japonsku a Koreji, pravděpodobně v důsledku lodní dopravy z Ameriky. V České republice je *P. magnifica* invazivním druhem, který se začal od roku 2003 masivně šířit na území Jižních Čech. Počet studií z různých zemí, které se zabývají invazivním šířením této mechovky, jejím vlivem na původní vodní ekosystém a zdraví lidí, stále narůstá. V tomto kontextu je považována za biologické znečištění, mezihostitele pro parazity ryb, nebo jako zdroj antimikrobiálních látek. Studie zaměřené na biologickou aktivitu sladkovodních mechovek jsou vzácné, a zatím nepřinesly žádné informace o konkrétní sloučenině. U mořských druhů byla ovšem popsána celá řada biologicky aktivních sekundárních metabolitů, které mechovkám slouží jako ochrana před predátory, parazity, infekcemi nebo jako chemikálie pro vnitrodruhovou a mezidruhovou komunikaci. Tímto ovlivňují množství mikro- i makroorganismů ve svém okolní. Kromě toho, některé z těchto látek mají unikátní vlastnosti, které mohou být využity pro farmakologické účely. Producenty biologicky aktivních látek ovšem nejsou v některých případech mechovky samotné, nýbrž jejich symbiotické nebo epibiotické bakterie. Ačkoliv bylo na *P. magnifica* pohlíženo z různých perspektiv, žádná ze studií se nezabývá bakteriemi s ní souvisejícími. Charakterizace bakterií z kolonií *P. magnifica*, a testování jejich antagonistické aktivity je dalším krokem k prohloubení vědomostí o případných interakcích této mechovky s původním ekosystémem.

2. Současný stav problematiky

2.1. Charakteristika kmenu Bryozoa (Ectoprocta)

V zahraniční literatuře lze Bryozoa najít také pod anglickým názvem "moss animals", což je doslovný překlad z řeckého bryon (mech) a zoon (živočich), jejichž spojením získáme název kmene (Russell 1965). Obdobný je i český ekvivalent, mechovky. Jedná se o velmi heterogenní skupinu vodních, modulárních, bezobratlých živočichů, kteří se vyskytují ve všech zeměpisných šířkách, hloubkách a stupních salinity, včetně sladkých vod (Waeschenbach et al. 2012). V současnosti Bryozoa zahrnují 3 třídy, 4 řády, 187 čeledí, 808 rodů a 5869 druhů. Z tohoto množství se pouze malá část nachází ve sladkovodních stanovištích. Sladkovodní jsou všechny druhy příslušné třídě Phylactolaemata, malé množství pak řádu Ctenostomata třídy Gymnolaemata, která v podstatě zahrnuje mořské druhy (Bock & Gordon 2013). Tito živočichové vytváří kolonie (zoaria) složené z identických, vzájemně propojených organismů (zoidů), připomínajících polypové stádium žahavců. Každý zoid je tvořen dvěma částmi: polypidem a cystydem. Polypidem je nazýváno vlastní tělo jedince se všemi orgánovými soustavami. Je zcela zatažitelný a nese vířivý aparát tzv. lofofor se svazkem dutých pohyblivých vláken. Cystyd je pevná nepohyblivá část, vytvářející oporu těla. V závislosti na druhu může být cystyd z organické hmoty, chitinový nebo vápenatý (Wood 2010). Tvar kolonií je velmi variabilní, od štíhlých rozvětvených tubulů, vláknitých struktur až po oválné útvary z gelového materiálu. Všechny mechovky jsou v podstatě přisedlé organismy, ale některé, jako například *Cristatella* nebo *Pectinatella*, mají schopnost mírného pohybu po substrátu (Ward & Whipple 1918, Massard & Geimer 2008). Nejdůležitějším zdrojem potravy je fytoplankon, který získávají filtrací vody pomocí ramen lofoforu. U mechovek je známo jak pohlavní, tak nepohlavní způsob rozmnožování. Upřednostňují však nepohlavní způsob tj. pučením. Sladkovodní druhy navíc vytvářejí vnitřní pupeny, statoblasty. Tato dormatní stádia umožňují mechovkám přežít nepříznivé teplotní podmínky, vysušení, průchod trávicí soustavou živočichů, a také šíření (Brooks 1929). Bryozoa významně přispívají k rozmanitosti druhů ve vodních ekosystémech. Tvoří nedílnou součástí bentické komunity, mimo jiné i tím, že slouží jako habitat pro řadu bezobratlých živočichů, jako jsou mechovnatci, korýši, juvenilní stádia mlžů, roztoči, houby, ploštěnci, nebo pláštěnci (Cocito 2004). Kromě toho poskytují potravu pro některé mikro predátory: ploštěnky, hlístice, mnohoštětinatce, různonožce, klanonožce, nohatky a nahožábré (Lidgard 2008).

2.2. Charakteristika *Pectinatella magnifica*

Pectinatella magnifica (bochnatka americká) je sladkovodní mechovka, která se řadí do třídy Phylactolaemata, řádu Plumatellida, čeledi *Pectinatellidae*. Tento druh poprvé objevil a popsal Leidy v roce 1851, poblíž Filadelfie (USA). Tvoří přisedlé žlutohnědé kolonie oválného tvaru na povrchu kamenů, větví nebo jiných ponořených substrátů, viz Obr. 1. Velikost kolonií se velmi liší. Průměrná hmotnost se pohybuje mezi 200 až 500 g, ale může dosahovat i desítek kilogramů (Balounová et al. 2011).

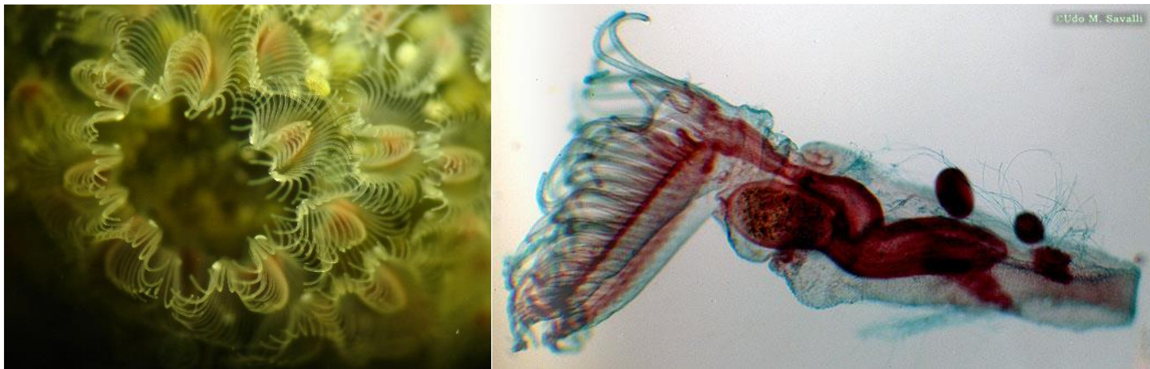
Obr. 1 Podélný řez kolonií *Pectinatella magnifica*

Foto: KMVD

Vnitřní část kolonií je tvořena hyalinní gelovou hmotou. Po chemické stránce je složena převážně z vody (99 %), dále pak z chitinu, vápníku, chloridu sodného a proteinů (Joo et al. 1992). Povrch je tvořen tenkou vrstvičkou zoidů, o velikosti 1 mm (Obr. 2), kteří produkují tuto hmotu, jako svou podkladovou bázi. Zoidi se seskupují se do růžencovitých útvarů tzv. roset po 12-18 jedincích. Na rozdíl od mořských druhů, mezi sebou jedinci v rosetách nemají příčky. Vzájemně sdílejí některé tkáně a tělní tekutiny, a vytváří tak fyziologicky jednotnou kolonii (Wood 2010). Mají podkovovitý tvar lofoforu, který je opatřen 80-84 řasinkovými chapadly (ciliemi). Ty jsou v neustálém pohybu, čímž umožňují proudění vody a přihánění potravy do ústního otvoru, který se nachází uvnitř lofoforu (Ingold et al. 1984). Trávicí soustava je ve tvaru U, takže řitní otvor vyúsťuje poblíž otvoru ústního (Brooks 1929). Životní cyklus zahrnuje pohlavní i nepohlavní rozmnožování. *P. magnifica* je hermafrodit, tentýž jedinec produkuje spermie i vajíčka, a dochází k vnitřnímu oplození. Z embrya se postupně vyvine larva, složená ze dvou plně vyvinutých zooidů a řasnatého pláště. Po určité době přisedá na vhodný substrát a pučením vytváří novou kolonii (Wood 2010). Bochnatka spíše upřednostňuje nepohlavní

způsob rozmnožování tj. pučením a tvorbou statoblastů. Statoblasty *P. magnifica* jsou čočkovitého tvaru a jejich chitinová stěna je na okraji naplněna vzduchem, což umožňuje plavání. Tento typ vnitřního pupenu je proto nazýván floatoblast. Na povrchu prstence vyrůstají háčky (11 – 22), které slouží především k uchycení na vhodný substrát a k šíření, pravděpodobně vodním ptactvem (Brooks 1929). Bochnatka americká se stejně jako ostatní kontinentální druhy vyskytuje ve všech typech vodních toků: rybníky, jezera, nádrže, řeky i ústí řek (Massard & Geimer 2008).

Obr. 2 Jednotliví zoidi *Pectinatella magnifica*

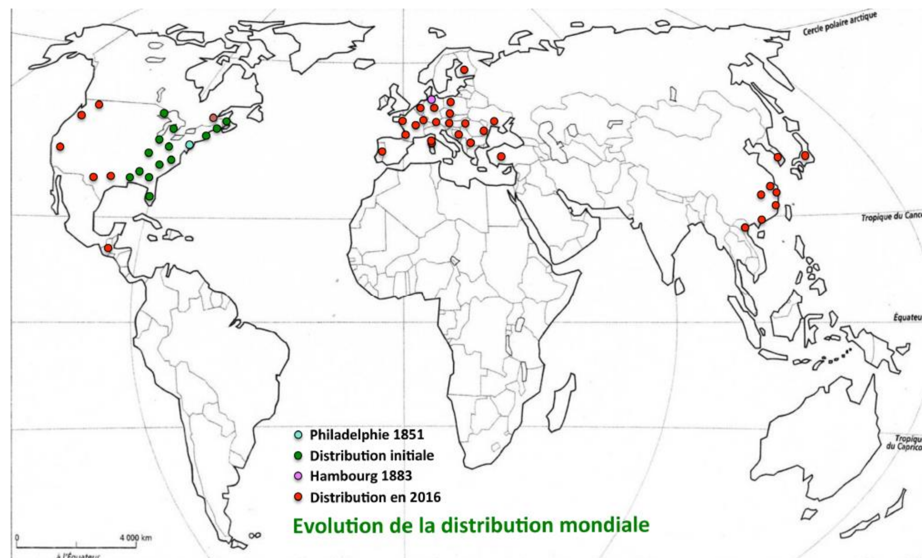


Dostupné z: www.gt-ibma.eu http://www.savalli.us

2.3. Šíření *Pectinatella magnifica* a její vliv na ekosystém

Původně se jedná o severoamerický druh, který byl prvně detekován v regionu poblíž Filadelfie (USA). Její výskyt byl ovšem záhy zaznamenán i v Evropě, Japonsku a Koreji. V těchto oblastech se jedná o nepůvodní druh, pravděpodobně zavlečený lodní dopravou z Ameriky a ponechaný v přístavu při čištění sladkovodních nádrží, ve kterých se nacházely statoblasty (vnitřní pupeny) a zoaria (kolonie) bochnatky. Tuto domněnku potvrzuje zjištění, že všechny lokality výskytu této mechovky v Evropě, se nacházejí podél lodních tras, odkud se mohly dále šířit (Lacourt 1968, Balounová et al. 2013). Kromě lokalit detailně popsaných v práci Balounová et al. (2013), byl její výskyt nově zaznamenán v roce 2013 i v Maďarsku (Szekeres et al. 2013), o rok později na Ukrajině (Aleksandrov et al. 2014), a v roce 2016 v Číně (Wang et al. 2017), viz Obr 3. Lze tedy předpokládat, že hlášení o jejím výskytu z dalších zemí bude i nadále přibývat.

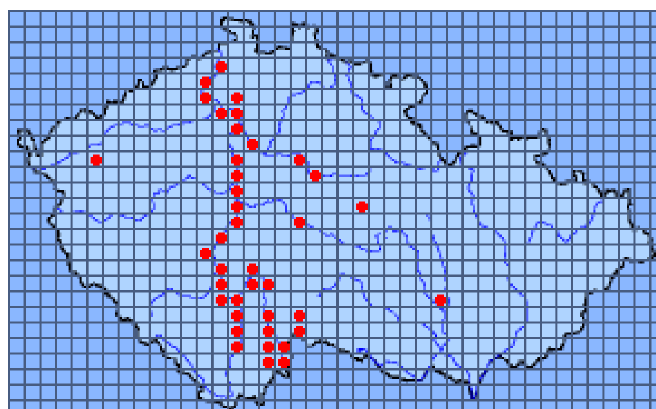
Obr. 3 Mapa vývoje šíření *Pectinatella magnifica*



Dostupné z: www.gt-ibma.eu

Prvně byla *P. magnifica* v České republice zaznamenána již před rokem 1952, od roku 2003 se však začala masivně rozšiřovat v Jižních Čechách, kde její výskyt nabyl invazivního charakteru (Balounová et al. 2011). Na internetových stránkách www.biolib.cz probíhá mapování jejího výskytu na našem území (Obrázek 4). Informace jsou průběžně aktualizovány, a to i ve spolupráci se širokou veřejností. Na stránkách je možné vyplnit záznam o místě pozorování, a aktivně se tak zapojit do monitoringu nepůvodních druhů na území České republiky.

Obr. 4 Mapa výskytu *Pectinatella magnifica* na území České republiky



Dostupné z: www.BioLib.cz

Vzhledem k jejímu masivnímu šíření, vyvstává otázka, jaký případný dopad může tato invaze mít na původní vodní ekosystém, činnost a zdraví člověka. V první řadě bývá považována za biologické znečištění (Wang et al. 2017). Její kolonie mohou dorůstat velkých rozměrů nebo vzájemně srůstat, a tím ucpávat potrubí a rošty vodních elektráren (Ingold et al. 1984). Dalším aspektem je vliv *P. magnifica* na původní mikro- a makroorganismy. Jakožto filtrátor zkrmuje plankton, drobné nálevníky a rozsivky (Lacourt 1968), čímž může docházet ke kompetici o živiny, případě ke změně chemismu vody produkcí primárních metabolitů. Setlikova et al. (2013) uvádí, že u většiny vodních ploch, kde se tato mechovka nachází, byla zaznamenána nižší koncentrace celkového fosforu, ve srovnání s vodními plochami typickými pro daný typ a oblast. Kolonie samotné také slouží jako ekologická nika s vyšší úrovní trofismu, oproti okolní vodě. Gelová hmota bývá osidlována některými prvoky, ploštěnkami, drobnými měkkýši a korýši. Rozkladný proces odumřelých těl těchto obyvatel mikroorganismy, pak láka nálevníky sloužící jako zdroj potravy pro polypidy (Lacourt 1968). Zejména na konci vegetačního období, kdy se kolonie začínají rozkládat, bývá v gelové hmotě detekováno také velké množství sinic, zelených řas a rozsivek (Setlikova et al. 2013). Právě kumulace toxigenních sinic by mohla představovat zdravotní riziko, zejména v rekreačních oblastech a chovných rybnících.

2.4. Biologicky aktivní látky produkované mechovci

Mořští bezobratlí živočichové jsou významnými producenty biologicky aktivních látek (Molinski et al. 2009). Přestože studium sekundárních metabolitů mechovek tvoří jen minoritní část publikací týkající se této problematiky, má velký potenciál (Sharp et al. 2007). Z více než 5000 recentních druhů bylo do roku 2010 studováno z hlediska produkce biologicky aktivních látek pouze 32, ty ovšem poskytly přibližně 200 různých sloučenin (Heindl et al. 2010). Chemická struktura těchto látek je velmi variabilní, od jednoduchých jedno nebo dvou uhlíkatých sloučenin až po komplexní makrocyklické sloučeniny. Jedná se především o steroly, terpeny, deriváty mastných kyselin, alkaloidy a makrolidické laktony (Blackman & Walls 1995). Mechovkám tyto látky slouží jako obranný systém proti konkurenčním organismům, predátorům, parazitům a infekcím, nebo jako chemikálie pro vnitrodruhovou a mezidruhovou komunikaci (Sinko et al. 2012). Konkrétním příkladem jsou amathamidy (bromované alkaloidy) produkované mechovkou *Amathia wilsoni*, které mají schopnost odradit ryby od jejího zkrmování (Sherwood et al. 1998), brom alkaloidy a diterpen z druhu *Flustra foliacea*, z nichž některé působí antimikrobiálně (Peters et al. 2003) nebo alternatamidy (bromtryptamin

peptidy) z *Amathia alternata* (Lee et al. 1997), taktéž s antimikrobiální aktivitou. Kromě baktericidní aktivity jako takové, zasahují některé z těchto metabolitů do buněčné komunikace tvz. quorum-sensing. Mnoho bakterií používají tento způsob komunikace pro regulaci svých počtů a funkcí (pohyblivost, faktory virulence nebo formace biofilmu). Inhibice systému pro quorum-sensing bez baktericidních účinků je velmi přínosná, protože zabraňuje rozvoji infekce/znečištění bez specifické blokace růstu. Vzhledem k tomu, že na bakteriální buňky není vyvíjen žádný selekční tlak, nevytváří se vůči těmto látkám rezistence (Sharp et al. 2007). Významnou skupinu biologicky aktivních sekundárních metabolitů mechovek tvoří bryostatiny. Jedná se makrolidické laktony, které jsou získávány extrakcí z kolonií *Bugula neritina* (Davidson & Haygood 1999). U nich byla prokázána celá řada biologických funkcí, viz kapitola 1.5. Ještě donedávna nebyly dostupné žádné práce, zaměřené na produkci biologicky aktivních látek sladkovodními mechovkami. Na ty se prvně zaměřil až srbský kolektiv, který se zabýval antimikrobiální aktivitou mechovky *Hyalinella punctata* (Pejin et al. 2012), jejím vlivem na tvorbu biofilmu a quorum-sensing *Pseudomonas aeruginosa* PAO1 (Pejin, Ciric, Karaman, et al. 2015), a prakticky simultánně s touto prací i na antimikrobiální aktivitu extraktů připravených z lyofilizovaných kolonií *P. magnifica* (Pejin, Ciric, Horvatovic, et al. 2015). Ačkoliv nebyla dosud izolována a charakterizována žádná konkrétní chemická sloučenina, extrakty obou výše zmíněných mechovek vykázali silnou antimikrobiální a antifungální aktivitu. Extrakt z *Hyalinella punctata* navíc inhibovala quorum-sensing potenciálně patogenní bakterie, *Pseudomonas aeruginosa* PAO1.

Producenty sekundárních metabolitů nemusí být pouze mechovky samotné. Mnoho produktů izolovaných z mořských makroorganismů se silně podobá těm, které byly již dříve identifikovány u bakterií. Tato skutečnost ve spojení s faktem, že vodní bezobratlí bývají hojně osidlováni symbiotickými mikroorganismy, vedla k myšlence, že některé z těchto látek jsou mikrobiálního původu (König et al. 2006). Touto hypotézou se ve své práci zabýval například Walls et al. (1995). Většina biologicky aktivních látek produkovaných mechovkou *Amathia wilsoni* má ve své struktuře zabodovaný brom. Práce tedy byla zaměřena na detekci tohoto prvku v různých částech kolonií. Vyšší koncentrace bromu byla zaznamenána nikoliv uvnitř mechovky, ale na povrchu kolonií. Navíc byla zjištěna korelace mezi hladinou bromu a výskytem bakterií ve tvaru tyčinek, což naznačovalo jistou souvislost. Skutečný důkaz pro tuto hypotézu poskytl Schmidt et al. (2000), který se zabýval lokalizací přírodních produktů u mořských hub. Zjistil, že cytotoxický makrolid swinholid A a peptid theopalauamid se

nacházejí v bakteriálních buňkách uvnitř *Theonella swinhoei*, zatímco frakce samotné houby žádné sekundární metabolity neobsahují.

2.5. Symbiotické mikroorganismy

Symbióza je termín, který bývá přijímán a chápan dvěma různými způsoby. Všeobecně jsou za symbiotické považovány bakterie žijící v rámci/uvnitř těla hostitele a zejména takové, žijící uvnitř hostitelské buňky (endosymbionti). Někteří autoři ovšem tento termín používají obecně, bez ohledu na to jaké mají bakterie na hostitele vliv a míru závislosti, jiní autoři zase speciálně pro mutualistický vztah, v pojetí evolučně-ekologickém (Moran & Wernegreen 2000). V této disertační práci rozumějme symbiotickým vztahem interakci dvou organismů bez blíže specifikovaného vymezení na úroveň komensalismu, mutualismu či parazitismu. Někteří z autorů uvedených níže ovšem pracují s druhým pojetím termínu. Takové mikroorganismy nelze kultivovat axenicky v podmínkách *in vitro*, a lze je tedy definovat pouze pomocí molekulárně-biologických metod. Pro bakteriální taxony, které nelze charakterizovat jinak, než pouze na základě genotypu, byla zavedena provizorní taxonomická kategorie tzv. „*Candidatus*“ (Murray & Stackebrandt 1995), a právě s tímto statusem se setkáváme v kontextu se symbiotickými mikroorganismy, jsou-li podrobněji definovány. V současné době je o symbiotických bakteriích mechovek jen velmi omezené množství informací. Ve většině případů nejsou jejich role v životech hostitelů dosud známy či uspokojivě objasněny.

Pukall et al. (2001) se zabýval kultivovatelnými bakteriemi mořské mechovky *Flustra foliacea*. Pomocí restrikční analýzy amplifikované ribosomální RNA (ARDRA) a genu pro 16S rDNA analyzoval bakterie z kolonií, pocházející ze dvou různých odběrových míst. Vzájemně také porovnával bakteriální diversitu kolonií odebraných ve stejné lokalitě, v různém čase. Nejčastěji detekované druhy byly *Shewanella frigidimarina*, *Pseudoalteromonas* ssp. a *Psychrobacter* ssp. Nalezeni byli také další zástupci kmenů *Proteobacteria, Cytophaga-Flavobacterium-Bacteroides* (CFB) a grampozitivní bakterie. Druhové zastoupení a počet jednotlivých taxonomických skupin se lišily, jak mezi koloniemi z různých odběrových míst, tak mezi koloniemi ze stejné lokality v různých časových úsecích. Mimo to, všechny izoláty patřily k druhům, zcela běžným pro mořské prostředí. Lze tedy konstatovat, že povrch *Flustra foliacea*, je kolonizován mikroorganizmy z okolí. Heindl et al. (2010) se zabýval kultivovatelnými bakteriemi 14 různých druhů mechovek obývajících Středozemní a Baltské moře. Na základě identifikace analýzou 16S rDNA zjistil, že kromě jediného, byly všechny izoláty příslušné gramnegativním třídám: *Flavobacteria*, *Alpha-* a *Gammaproteobacterium*, a

rodům: *Cellulosimicrobium*, *Tenacibaculum*, *Pseudovibrio*, *Shingomonas*, *Alteromonas*, *Marinomonas*, *Pseudoalteromonas*, *Pseudomonas*, *Shewanella* a *Vibrio*. Dále byla testována jejich antimikrobiální aktivita. Ta byla prokázána u 101 izolátů, z celkového počtu 340 izolátů. Antimikrobiálně působily převážně proti grampozitivním bakteriím. Z ústí chitinózních schránek mechovky *Myriapora truncata* byly izolovány nepravidelné tyčinky se schopností bioluminiscence. Na základě fenotypové a genotypové analýzy byly identifikovány, jako nový druh *Vibrio* sp. MT1 (Stabili et al. 2008). Autoři předpokládají vysoce specifický vztah tohoto mikroorganismu s hostitelem, pro hypotézu zatím ale neposkytli přesvědčivý důkaz. Vzhledem k oportunní patogenitě některých vibrií a hojnému výskytu mechovek, by jejich vzájemné interakce mohly mít i případný epidemiologický a ekologický dopad. U *Bugula neritina* byl zjištěn symbiotický mikroorganismus "*Candidatus* Endobugula sertula", řazený mezi γ-proteobakterie. "*Candidatus* E. sertula" je skutečných producentem výše zmíněných bryostatinů, ty slouží jako chemická obrana hostitele (Davidson & Haygood 1999). V larvách příbuzného druhu mechovek *Bugula simplex* byly objeveny bakterie ve tvaru dlouhých tyčinek a na základě sekvence části genu pro 16S rRNA pojmenovány jako "*Candidatus* Endobugula glebosa". "*Candidatus* E. glebosa" produkuje látku obdobného charakteru jako je bryostatin (Lim & Haygood 2004), ta slouží k ochraně larev mechovky před predátory (Lim-Fong et al. 2008). U mechovek rodu *Watersipora* byla pomocí molekulárně genetických metod zjištěna přítomnost symbiotických α-proteobakterií, s pleomorfním tvarem. V larvách mechovky byly detekovány ihned po jejich uvolnění do vodního sloupce, což značí vertikální přenos. Pro symbionta druhu *W. subtorquata* byl na základě sekvence 16S rDNA navržen název "*Candidatus* Endowatersipora palomitas" a pro symbionta druhu *W. arcuata* název "*Candidatus* Endowatersipora rubus" (Anderson & Haygood 2007).

O symbiotických bakteriích sladkovodních mechovek bohužel žádné informace dostupné nejsou. Sladkovodní druhy, byly nicméně studovány z pohledu mezihostitele parazitů ryb, jmenovitě mikrosporidií. Byla u nich nalezena a popsána celá řada nových taxonů těchto diplokaryotních organismů. Příkladem je *Schroedera plumatellae* gen.nov., sp.nov. z mechovky *Plumatella fungosa* (Morris & Adams 2002). Canning (et al. 2002) popsal na základě morfologických znaků a analýzy rDNA tři nové druhy. *Bryonosema plumatellae* gen.nov., sp.nov z *Plumatella nitens*, *Bryonosema tuftyi* z *Plumatella* sp. a *Trichonosema pectinatellae* gen.nov., sp.nov. z mechovky *Pectinatella magnifica*. O dva roky později byl u *P. magnifica* popsán další nový druh, *Trichonosema algonquinensis* (Desser et al. 2004).

2.6. Potenciální využití sekundárních metabolitů

Vzhledem k tomu, že sekundární metabolity mechovek, případně jejich symbiotických bakterií, mají antagonistické vlastnosti, byla provedena řada studií za účelem zjištění jejich možného dopadu na lidské zdraví. Testování bylo zaměřeno především na látky s cytotoxickým a antibiotickým účinkem (Heindl et al. 2010). Bioaktivní alkaloidy pteroceliny, produkované mechovkou *Pterocella vesculosa* vykazují, díky svým cytotoxickým vlastnostem, silnou aktivitu proti leukemickým buňkám myší. Jsou také považovány za možné terapeutikum pro léčbu nádorových onemocnění a vykazují silnou aktivitu proti grampozitivní bakterii *Bacillus subtilis* a plísni *Tricophyton mentagrophytes* (Prinsep et al. 2004). Podobné účinky byly zjištěny i u extraktu z mechovky *Caulibugula intermis*, který při testování *in vitro* působil proti nádorovým buňkám myší. Příčinou cytotoxické aktivity jsou bioaktivní látky získané frakcionací extraktu, zvané caulobugulony A-F (Milanowski et al. 2004). *Biflustra perfragilis* je producentem isochinolinových chinonů, perfragilinu A a perfragilinu B. Oba působí cytotoxicky na lidské rakovinné buněčné linie (Sharp et al. 2007). Největší potenciál pro farmakologické využití mají bryostatiny. Pro své cytotoxické vlastnosti je zejména bryostatin 1 předmětem výzkumu jako možné terapeutikum při léčbě nádorových onemocnění, nebo prostředek pro posílení paměti. Dále se uvádí, že je v subnanomolárních koncentracích silným aktivátorem proteinkinázy C (Pettit & Herald 1982, Hennings et al. 1987) a může být uplatněn při léčbě a prevenci HIV-1 (DeChristopher et al. 2012).

2.7. Metody studia diversity bakteriálních společenstev

Existuje celá řada přístupů, metod a technik, kterými lze charakterizovat mikroorganismy osidlující určitou ekologickou niku. Na jedné straně stojí metody klasické, na straně druhé metody moderní -omické (omickými metodami rozumějme nástroje pro studium genů - genomika, mRNA - transkiptomika, proteinů - proteomika, a metabolomu - metabolomika).

Z historického hlediska sahají počátky identifikace a taxonomického řazení do konce 19. století, kdy se opíraly výhradně o fenotypové charakteristiky. V této době byly nejdůležitějšími znaky pro klasifikaci morfologie, růstové požadavky a patogenní potenciál. Mnoho dnes známých patogenních druhů bakterií bylo popsáno právě v letech 1880 až 1900. Na počátku 20. století se k morfologickým znakům přidaly i znaky fyziologické a biochemické. Později byly zkoumány enzymy a objasněny metabolické dráhy. Od 60. let 20. století se mikroorganismy

začaly klasifikovat i podle znaků chemotaxonomických, a podle genotypu (Schleifer 2009). V 80. letech 20. století objevili vědci rozpor v počtu bakteriálních buněk při přímém pozorování pod mikroskopem a počtu kolonií narostlých na kultivačním médiu. Tento jev, nazývaný „great plate count anomaly“, byl první známkou faktu, že v laboratorních podmínkách lze kultivovat jen některé mikroorganismy. Toto zjištění přispělo k rozvoji kultivačně nezávislých molekulárně-biologických metod (Uhlík et al. 2013). Uvádí se, že relativní podíl bakterií rostoucích v *in vitro* podmínkách (Kolonie Tvořící Jednotky- KTJ, stanovené jako procento kultivovatelných bakterií ve srovnání s celkovým množstvím) je 0,1 - 1 % v nedotčených lesních půdách, kolem 10 % v prostřední jako je orná půda, 0,001 - 3 % ve vodě, 1 - 15 % v aktivovaném kalu, a 0,25 % v sedimentu (Torsvik et al. 1990, Amann et al. 1995, Cottrell & Kirchman 2000). V lidském trávicím traktu bylo v 70. letech 20. století kultivačně stanoveno a identifikováno více než 400-500 různých druhů bakterií, přesto se odhaduje, že 60 - 80 % z celkové mikrobiální diverzity je běžnými *in vitro* technikami nedetekovatelných (Dave et al. 2012). Naproti tomu, moderní sekvenační metody poskytují přesné a detailní informace o skutečné mikrobiální diversitě. Díky těmto metodám je možné detekovat nejen mikroorganismy, které nejsou schopné růstu v experimentálních podmínkách, ale také pomalu rostoucí mikroorganismy a mikroorganismy vyskytující se v přírodě v nízkých počtech, tudíž prakticky neizolovatelné. Na druhou stranu, izolace mikroorganismů v čisté kultuře je první krok k identifikaci nových druhů. Kultivace umožňuje studium celého organismu, včetně fenotypu, a poskytuje prostředky k ověření hypotéz vyplývajících z genomických dat. Z axenické kultury je také možné izolovat kompletní genom o vysoké kvalitě, který může sloužit jako referenční pro různé meta-omické metody, a pro vytvoření specifických primerů a sond. I v současné „omické“ době tedy zůstávají klasické techniky základním nástrojem v mikrobiologii, a stále vznikají studie, které se odvolávají na potřebu izolace a kultivace mikroorganismů (Giovannoni & Stingl 2007, Garcia 2016, Salcher & Šimek 2016).

2.7.1. Kultivační metody

Kultivační metody se opírají o specifická pěstební média sloužícím k vyčíslení a izolaci životaschopných bakteriálních buněk. Složení kultivačních médií pak závisí na účelu studia. K získání co nejširšího spektra mikroorganismů z environmentálního prostředí se používají elektivní média s bohatým zdrojem živin. Přesné složení by pak mělo korespondovat s množstvím a druhovým zastoupením živin, přirozeně se vyskytujícím ve vzorku (Mandal et al. 2011). Naopak, k detekci určité skupiny mikroorganismů jsou používána média se

specifickým složením živin, energetických zdrojů a selektivních antimikrobiálních látek (Vlková et al. 2015). Vysoce specifická jsou chromogenní média, která jsou vyvinuta pro stanovení cílových mikroorganismů bez potřeby následné identifikace. Využívají enzymaticky specifické substráty s navázanou chromogenní látkou. Při hydrolýze uvolňují barvivo a cílené mikroorganismy tvoří barevné kolonie, snadno odlišitelné od jiných druhů. V ideálním případě by měl být růst jiných, než cílových druhů, zcela inhibován (Perry & Freydière 2007). Kultivační techniky představují velmi citlivou, finančně nenáročnou metodu, která poskytuje jak kvalitativní, tak kvantitativní informace o počtu a povaze mikroorganismů přítomných ve vzorku (Mandal et al. 2011). Umožňují namnožit mikrobiální materiál a získat jednotlivé mikroorganismy (čisté kultury) z jediné mateřské buňky ve vzorku. Sériové ředění a následné množení mikroorganismů v přítomnosti různých zdrojů energie, stanovení makromolekulárního složení buněk, vedlejší produkty metabolismu a použití specifických imunologických činidel se staly základem identifikace a třídění mikroorganismů (Relman 1998, Mandal et al. 2011). Kromě faktu, že *in vitro* lze detekovat pouze minoritní část mikrobiální populace, je kultivace časově náročná a náročná na pracovní sílu. Další omezení analýzy spočívají v nerovnoměrném zastoupení mikroorganismů ve vzorku, heterogenitě matrice, formě vzorku (prach, kapalina, gel atd.) nebo v rozdílné viskozitě způsobené přítomností tuků a olejů, které negativně ovlivňují homogenizaci (Mandal et al. 2011). U environmentálních vzorků představují problém uvolňování bakterií či spor z matrice a biofilmů, selektivita kultivačního média, nebo rozdílná růstová rychlost mikroorganismů (Kirk et al. 2004).

2.7.2. Metody biochemické a chemotaxonomické

2.7.2.1. Fyziologický profil

Jedním ze základních přístupů k identifikaci mikroorganismů je zjišťování metabolických drah nebo vybraných enzymů testovaných mikroorganismů. Ty jsou kultivovány za přítomnosti různých druhů uhlíku a indikátoru v mikrotitračních destičkách. V případě, že je daný substrát metabolizován, jeho degradace je provázena barevnou změnou indikátoru. V současné době je na trhu dostupná celá řada komerčně vyráběných souprav, které slouží ke stanovení biochemických profilů. Většina z nich je určena pro analýzu čistých kultur, jsou ovšem dostupné i testy pro stanovení funkčních aspektů na úrovni společenstev (Stefanowicz 2006). První z těchto aspektů je funkce komunity, tedy skutečná katabolická aktivita. Druhým aspektem je funkční diverzita, tedy schopnost komunity přizpůsobit metabolismus (katabolismus), a/nebo relativní složení a velikost populace v různých

abiotických podmínkách (mikroklima, přídatné substráty atd.). Nezbytné jsou informace o obou těchto aspektech (Preston-Mafham et al. 2002). Prvně byl pro účely studia fyziologického profilu na úrovni celé komunity použit systém Biolog, Inc., původně určený pro identifikaci čistých kultur. Do mikrotitrační destičky obsahující 95 různých zdrojů uhlíku byl inokulován celý environmentální vzorek a inkubován při konstantní teplotě. Vyhodnocení je založené na výměně elektronů během respirace (tvorba NADH), což vede k redukci a barevné změně přítomného indikátoru tetrazoliové violeti (není redukovatelná houbami). Změna barvy je pak stanovena spektrofotometricky. Míra využití substrátů různými skupinami mikroorganismů se liší, takže je možné pozorovat vysokou proměnlivost v rychlosti a intenzitě vývoje barvy, v závislosti na složení mikrobiální komunity. Tímto způsobem lze získat takzvaný metabolický fingerprint (Garland & Mills 1991). V současné době jsou vyráběny testy určené speciálně pro ekologické studie mikrobiálních společenstev. Takovým testem je například Eco plates (Biolog, Inc.), kde desky obsahují 31 substrátů ve třech opakováních, což také umožňuje statistickou analýzu (Stefanowicz 2006). Tato metoda je vhodná zejména pro hodnocení relativních rozdílů ve funkční diverzitě (roli jednotlivých složek) mikroorganismů např. v rostlinné rhizosféře nebo kontaminovaných lokalitách. Nicméně i tato metoda má svá omezení: selektuje pouze mikroorganismy, které jsou schopné růstu v experimentálních podmínkách, zvýhodňuje rychle rostoucí organismy a je citlivá na hustotu inokula. Kromě toho zdroje uhlíku v destičkách nemusí nutně odpovídat těm, které jsou přirozeně přítomné ve vzorku (Kirk et al. 2004).

2.7.2.2. Analýza mastných kyselin

Mastné kyseliny jsou, jakožto složka membránových lipidů, nepostradatelnou součástí každé živé buňky. Mají velmi rozmanitou strukturu a zároveň jsou vysoce biologicky specifické, proto slouží jako biomarkery pro studium mikroorganismů. Analýza mastných kyselin se využívá jak pro identifikaci čistých kultur a taxonomické třídění, tak pro studium mikroorganismů z environmentálních vzorků nebo laboratorně kultivovaných směsných kultur (Zelles 1999).

Často využívanou metodou je FAME (fatty acid methyl ester analysis). Jedná se o rychlou techniku založenou na čtyřech základních krocích: extrakci mastných kyselin z buněčných lipidů, metylaci mastných kyselin, extrakci metylesterů mastných kyselin a jejich následné analýzy na plynovém chromatografu. Buněčné lipidy jsou přítomné jak v živých, tak v mrtvých buňkách. U environmentálních studií musí být také bráno v úvahu, že v komplexních vzorcích jsou přítomné jak intracelulární, tak extracelulární lipidy. Extracelulární lipidy mohou

přetrvávat v organické matrici vzorku ve stabilních formách, tudíž výsledky analýzy neodráží aktuální diversitu, ale spíše poskytují vhled do její historie. Mimo to, mohou být odvozené nejen od bakterií, ale také eukaryotických mikroorganismů a makroorganismů (Haack et al. 1994, Zelles 1999).

Obdobou je extrakční a separační technika k analýze mastných kyselin odvozených od fosfolipidů, PLFA (phospholipid fatty acid analysis). V první fázi jsou pomocí pufru a vhodných rozpouštědel extrahovány všechny lipidy z buněčných struktur ve vzorku a následně rozděleny na frakce: neutrální lipidy, glykolipidy a fosfolipidy (polární lipidy). Z fosfolipidů jsou dále uvolněny mírnou alkalickou metanolýzou mastné kyseliny, převedeny na metylestery a analyzovány pomocí plynové chromatografie nebo hmotnostní spektrometrie (GC/MS) (Zelles 1999, Gattinger et al. 2003). Fosfolipidy jsou základní složkou všech buněčných membrán. Jako markery pro charakterizaci mikrobiálních společenstev jsou využívány díky specifitě mastných kyselin z nich odvozených, které se významně liší mezi jednotlivými mikrobiálními skupinami. Vzhledem k tomu, že po smrti rychle degradují a nevyskytují se v zásobních látkách, jejich analýza poskytuje informaci o skutečné diverzitě. Metoda FAME je na rozdíl od PLFA rychlejší a méně náročná, ale poskytuje méně spolehlivé výsledky (Makula 1978, Gattinger et al. 2003).

Obě výše zmíněné metody jsou vhodné pro studium bakterií a eukaryotických mikroorganismů, které mají mastné kyseliny vázané esterovou vazbou. Archaea mají mastné kyseliny vázané etherovou vazbou, tudíž nejsou hydrolyzovány pomocí standardních protokolů PLFA a FAME. Polární lipidy Archaea (etherové fosfolipidy) jsou jedinečné a snadno odlišitelné od fosfolipidů bakterií a eukaryot. Jsou složené z di- a tetraetherů glycerolu nebo složitějších vícesytných alkoholů s postranními izoprenoidními řetězci, které mohou být z vazby vyvázány pouze pomocí silných kyselin, jako je kyselina jodovodíková (Gattinger et al. 2003). Metoda pro jejich analýzu se nazývá PLEL (phosholipid etherlipid analysis).

Další metodou využívající buněčných lipidů je analýza izoprenoidních chinonů (quinonů). Metoda je založena na extrakci chinonů ze vzorku nepolárními (organickými) rozpouštědly, purifikaci a analýze za využití různých chromatografických metod, ultrafialové spektrofotometrie, nebo hmotnostní spektrometrie. Je aplikovatelná na všechny vzorky s obsahem mikrobiální biomasy $\geq 10^9$, a kombinaci s molekulárními technikami by měla poskytnout přesná a spolehlivá data o populační dynamice a struktuře společenstva (Collins & Jones 1981, Hiraishi 1999, Schleifer 2009). Izoprenoidní chinony hrají významnou roli při elektronovém transportu, oxidativní fosforilaci a pravděpodobně i aktivním transportu. Tvoří

tedy součást jak respiračního, tak fotosyntetického systému mikroorganismů. Podle stavební struktury se rozeznávají dvě základní skupiny chinonů: naftochinony a benzochinony. Význam pro klasifikaci mikroorganismů nemá jen typ chinonu (ubichinon, menachinon, atd.), ale také délka a saturace polyprenylových postranních řetězců.

Kromě výše zmíněných jsou popsány i další metody, mezi které patří například analýza kyseliny muramové, kyseliny teichoové, nebo mastných kyselin lipopolysacharidů charakteristických pro gramnegativní bakterie a sinice (Gehron et al. 1984, Vestal & White 1989).

Informace o složení komunity jsou založeny na frekvenci a poměru mezi jednotlivými mastnými kyselinami, které tvoří relativně konstantní část buněčné biomasy. Takže změny v profilech mastných kyselin představují změny v mikrobiální populaci. Na rozdíl od stanovení fyziologického profilu není analýza mastných kyselin závislá na kultivaci (Kirk et al. 2004). Při identifikaci druhů v rámci společenstva je však vyhodnocení softwarem omezeno právě na referenční profily čistých kultur kultivovaných v laboratorních podmínkách. Ne všechny druhy jsou touto metodou detekovatelné. Některé mikroorganismy nemají dostatečně charakteristické složení, aby byly rozpoznatelné z profilu celého společenství. Dalším důvodem může být rozdílná výtěžnost mastných kyselin (přibližně pětinásobná) napříč taxony (Haack et al. 1994, Zelles 1999).

2.7.2.3. Analýza proteinů

Dalším přístupem ke studiu mikroorganismů je analýza proteinů. Proteiny jsou nejhojnější a funkčně nejrozmanitější molekuly v živých systémech. Některé z proteinů jsou naprosto unikátní a specifické pro určité druhy, proto se využívají jako biomarkery. Vzhledem k tomu, že koordinace syntézy proteinů probíhá na ribozomech, byly jako vhodné biomarkery pro chemotaxonomickou charakterizaci zvoleny právě proteiny ribozomální (Teramoto et al. 2007).

Komplexním složením proteinů se zabývá vědní obor zvaný proteomika, který je obecně definován jako analýza kompletní sady bílkovin (proteomu) v buňce v daném okamžiku a za definovaných podmínek. Termín metaproteom pak představuje složení proteomu celé mikrobioty osidlující určitou niku, tedy smíšené mikrobiální populace (Wilmes & Bond 2006). Metaproteomika je všeobecně přijímána, jako klíčová technika pro sledování globální syntézy proteinů a genové exprese. V tomto kontextu, by měli studie poskytnout informace, umožňující nahlédnout do funkční dimenze genomového souboru, a tím přispět k dosažení hlavního cíle

environmentální mikrobiologie: schopnost spojit jednotlivé bakteriální druhy k jejich funkci v přírodě (Cash 2000, Wilmes & Bond 2006). Jinak řečeno, analýza metaproteomu umožňuje studium nových funkčních genů a metabolických drah, a identifikaci proteinů souvisejících se specifickým stresem (Maron et al. 2007). Samotný proces analýzy je technicky náročný a zahrnuje různé kroky od extrakce proteinů, stanovení jejich diverzity až po identifikaci. V prvním kroku jsou rozrušeny buněčné struktury. Lýza může probíhat buď přímo v matrici vzorku, nebo nepřímo. U nepřímé strategie jsou ze vzorku nejdříve extrahovány mikroorganismy, a z nich následně proteiny. Právě proces extrakce proteinů je v metaproteomice zásadní. Bývá negativně ovlivněn heterogenitou environmentálních vzorků, přítomností interferenčních sloučenin i samotnou komplexitou populací (Wilmes & Bond 2006). Separace a analýza proteinového mixu pak může být uskutečněna řadou různých biochemických metod. Obvykle se využívá elektroforetická či chromatografická separace v kombinaci s identifikací proteinů pomocí hmotnostní spektrometrie (MS). Za tradiční způsob separace se považuje dvoudimenzionální (2D) gelová elektroforéza, kdy jsou proteiny separovány na základě izoelektrické fokusace (IEF) v dimenzi první a molekulové hmotnosti v dimenzi druhé (SDS-PAGE). Díky 2D elektroforéze je možné rozptýlit velké množství proteinů včetně jejich post-translačních modifikací a poskytují tzv. „proteofingerprint“, který lze dále analyzovat počítačovým softwarem, a zjistit jím změny v genové expresy mezi vzorky. Konkrétní proteiny je pak možné z gelu vyříznout a identifikovat. Technologie na bázi chromatografie však bývají často upřednostňovány, jakožto spolehlivější separační nástroje (Wilmes & Bond 2004, Maron et al. 2007). Jak již bylo výše řečeno, pro identifikaci proteinů se pak používá řada různých metod založených na hmotnostní spektrometrii (MS). Velký pokrok v tomto směru nastal s nástupem tzv. měkkých ionizačních technologií, jmenovitě se jedná o elektrosprejovou ionizační (ESI) hmotnostní spektrometrii a hmotnostní spektrometrie s laserovou desorpcí a ionizací za účasti matrice s průletovým analyzátorem (MALDI-TOF) (Graham et al. 2007). Zejména MALDI-TOF MS je široce uplatňovaná metoda nejen pro environmentální analýzy, ale především pro rutinní klasifikaci a identifikaci bakterií, na základě charakterizace ribozomálních proteinů jako biomarkerů, v mnoha oblastech včetně potravinářského průmyslu a veřejného zdraví (Teramoto et al. 2007).

Proteomika poskytuje kvalitativní údaje o proteinech kódovaných bakteriálními genomy spolu s kvantitativními údaji o odezvě syntézy bílkovin za definovaných podmínek prostředí (Cash 2000). Použití tohoto přístupu k objasnění funkčních složek mikrobiálních

ekosystémů má obrovský potenciál, přesto však představuje velkou výzvu (Wilmes & Bond 2006).

2.7.3. Molekulárně-genetické metody

Základním předpokladem molekulárně-genetických metod je práce s nukleovými kyselinami. Zatímco DNA představuje veškerou genetickou informaci ve vzorku (genom - jediný organismus, metagenom - společenství) včetně komponent, které nejsou živé, RNA je syntetizována pouze aktivně rostoucími buňkami a relativně rychle degraduje. Messenger RNA (mRNA) bývá zpravidla stabilní jen velmi krátce, informuje tedy o genech, které byly aktivní v době extrakce. Oproti tomu ribozomální RNA (rRNA) je díky sekundární struktuře a vazbě na ribozomální proteiny stabilnější, takže může teoreticky zůstat v odumřelých buňkách neporušená i celé měsíce. Přesnější informace týkající se konkrétních funkcí lze získat z mRNA, práce s ní ale daleko obtížnější. Do formy DNA mohou být mRNA i rRNA převedeny pomocí reverzní transkriptázy (RT) (Hirsch et al. 2010). Nejlépe charakterizovaná molekula, která se používá v mikrobiální systematice je malá podjednotka rRNA (16S rRNA u prokaryot, 18S rRNA u eukaryot). Tato molekula je charakteristická pro všechny organismy, je konstantní, sekvence mají pomalou evoluční rychlost a počet mutací odpovídá evoluční vzdálenosti mezi kmeny, navíc obsahuje vysoce konzervativní úseky napříč říší s variabilními úseky specifickými pro určitý taxon. Další výhodou je její snadná izolace (Fox et al. 1980, Uhlík et al. 2013). Analýza genů pro 16S rRNA poskytuje rámec pro zařazení sekvencí na úroveň rodů či druhů. Pro odlišení nižších taxonomických podjednotek, případně některých blízce příbuzných druhů, musí být použity další markery. Pro tyto účely se využívají protein-kódující geny, které se vyznačují vyšší sekvenční variací. Jejich výhodou je také fakt, že se na rozdíl od 16S rRNA vyskytují v genomu pouze v jedné kopii, tudíž poskytují spolehlivější výsledky o relativní četnosti organismu (Hirsch et al. 2010). S nástupem molekulárně-genetických metod bylo vědcům umožněno studium skutečné mikrobiální diversity (Forney et al. 2004). Představují kultivačně nezávislý přístup, který se vyznačuje vysokou specifitou a citlivostí. Detekční limit pro tyto metody se uvádí zhruba 10^3 buněk/g (ml) vzorku, v závislosti na konkrétní metodě (Mandal et al. 2011). Běžně se používají pro identifikaci mikroorganismů, stanovení fylogenetické pozice neznámých druhů a pro studium diversity mikrobiálních komunit (Wintzingerode et al. 1997, Zhang et al. 2002).

2.7.3.1. *Izotopové značení*

Tato technika je přechodem mezi metodami biochemickými a metodami využívající analýzu nukleových kyselin. Izotopové značení představuje kultivačně nezávislé prostředky umožňující studium biochemických procesů, na kterých se podílejí mikroorganismy, čímž získáme odpověď na otázku: které funkce lze připsat kterým mikroorganismům v přirozeném prostředí. Tyto metody využívají fyzikálních vlastností atomů prvků, které jsou součástí všech buněčných struktur, především atomu uhlíku. Příkladem je technika SIP (stable-isotope probing), která využívá substráty značené stabilními izotopy. Tyto substráty jsou po inkubaci ve vzorku metabolizovány určitými skupinami mikroorganismů a zabudovány do buněčných struktur. Pro identifikaci mikroorganismů zapojených do biochemické transformace značeného substrátu může posloužit jako biomarker DNA, RNA nebo PLFA (mastné kyseliny odvozené od fosfolipidů). Pro značení DNA se využívají například stabilní izotopy ^{15}N a ^{14}N (NH_4Cl), nebo těžké stabilními izotopy ^{13}C a ^{2}H. Zabudování značeného izotopu do DNA značně zvyšuje rozdíl v hustotě značených a neznačených frakcí. Těžké (značené) a lehké frakce jsou od sebe oddělitelné hustotní gradientovou centrifugací. I velmi malé rozdíly v izotopovém poměru je následně možné detekovat hmotnostní spektrometrií izotopových poměrů (IMRS) nebo hmotnostní spektrometrií atomárních a molekulárních iontů (SIMS) (Radajewski et al. 2003).

2.7.3.2. *Hybridizace a reasociace nukleových kyselin*

Jednou z metod využívaných pro studium příbuzenských vztahů mikroorganismů je DNA-DNA hybridizace. Tato je metoda založená na denaturaci a reasociaci (renaturaci) dvoušroubovicové DNA. Při zahřátí na 100 °C dochází k rozpojení vodíkových vazeb a ke vzniku jednořetězcového vlákna. Následné ochlazení umožní opětovné spojení dvou řetězců. Fragmenty DNA dvou různých druhů mohou být smíchány, denaturovány a posléze vzájemně reasociovány za vzniku heteroduplexů (Bledsoe & Sheldon 2009). Genetická příbuznost mezi bakteriálními kmeny je hodnocena množstvím vzájemně spojených nukleotidových bází (Ezaki et al. 1989). Wayne et al. (1987) uvádí, že mikroorganismy jsou stejného druhu, pokud jsou jejich DNA k sobě komplementární nejméně ze 70 % a zároveň rozdíl v teplotě tání není vyšší než 5 °C. Proces hybridizace je také mnohdy časově náročný a náročný na pracovní sílu, navíc vyžaduje velké množství DNA o vysoké kvalitě. Hlavní nevýhodou vzhledem k srovnávací povaze této techniky je, že nelze vybudovat databáze, jako je tomu u sekvencí nukleotidů (Goris et al. 2007). Vzhledem k obrovské diversitě mikrobiálních populací není tato technika vhodná pro studium environmentálních vzorků. Genetickou rozmanitost bakteriálních společenstev

umožnuje posoudit reasociace denaturované DNA, kdy je ze vzorku extrahována celková DNA, naštěpena, denaturována a následně reasociována. Rychlost hybridizace nebo reasociace je závislá na podobnosti přítomných sekvencí. Se zvyšující se diversitou rychlost reasociace klesá. Za přesně definovaných podmínek může být čas potřebný k dosažení poloviční reasociace využit jako index diversity společenství. Případně je touto metodou možné studovat podobnost komunit v různých vzorcích měřením stupňů podobnosti na základě hybridizační kinetiky. Vyšší rozlišení a informace o změně v hrubé struktuře komunity však poskytují metody založené na polymerázové řetězové reakci (Torsvik et al. 1998).

Nejjednodušším způsobem, jak detekovat specifickou sekvenci nukleových kyselin je přímá hybridizace sondy na denaturované jednovlákenné nukleové kyseliny cílených organismů. Provádět je lze na extrahované DNA, RNA, nebo *in situ*. Jako sondy jsou používány oligonukleotidy (méně než 20 nukleotidů) nebo polynukleotidy (více než 50 nukleotidů), sestavené ze známých sekvencí s různou specifitou od domény až po druhovou úroveň (Ezaki et al. 1989, Kirk et al. 2004). Příkladem je flourescenční *in situ* hybridizace (FISH). Principem je hybridizace (navázání) fluorescenčně značené sondy na rRNA a jejich následná vizualizace prostřednictvím epifluorescenční a konfokální laserové skenovací mikroskopie, nebo průtokové cytometrie. Byly také popsány techniky, které využívají polynukleotidové DNA sondy nebo oligonukleotidové mRNA sondy. FISH se používá pro kvantitativní a kvalitativní stanovení mikroorganismů, a pro studium prostorové a časové dynamiky jednotlivých populací v jejich přirozeném prostředí. Při identifikaci mikroorganismů z environmentálních vzorků však představuje problém vytvoření specifických sond pro společenství o neznámém složení (Pernthaler et al. 2001).

Významnou metodou je DNA microarray hybridizace (neboli DNA mikročipy), která byla poprvé popsána v roce 1995 pro simultánní analýzu rozsáhlých genových expresí. Jedná se o soubor velkého množství DNA sond, cDNA nebo oligonukleotidů specifických pro cílený gen, DNA sekvenci nebo RNA sekvenci, uchycených na pevném nosiči, ke kterým se hybridizují komplementární značené fragmenty ze vzorku. DNA mikročipy mají velmi široké uplatnění. Kromě toho, že jsou uplatňovány pro studium mikrobiálních populací, jsou schopné detekovat i virovou DNA nebo specifické geny (např. geny kódující rezistenci k antibiotikům). Využívány jsou i při kontrole kvality potravin, diagnostickým a klinickým účelům (Hadidi et al. 2004).

2.7.3.3. Metody založené na polymerázové řetězové reakci (PCR)

Polymerázová řetězová reakce je metoda, která umožňuje *in vitro* amplifikovat specifický úsek DNA v prakticky neomezeném množství (více než 10^9 kopií během 30 cyklů) (Hirsch et al. 2010). Požadovaný fragment DNA nebo RNA je vymezen dvěma primery (oligonukleotidy), které jsou komplementární k sekvencím na okrajích množeného úseku. V závislosti na volbě primerů je možné detekovat buď funkční geny, nebo sekvence specifické pro určitou skupinu mikroorganismů (např. druhově, rodově specifické primery). Reakce je založena na třech jednoduchých krocích. Nejprve dochází k denaturaci DNA zahřátím, zpravidla na teplotu 90 - 95 °C. V dalším kroku tvz. annealingu se sníží teplota (45 - 60 °C), což umožní hybridizaci primerů na příslušné sekvence každého původního vlákna. V poslední fázi je teplota opět mírně zvýšena (70 - 72 °C), pro docílení optimální polymerázové aktivity. V tomto kroku zvaném extenze dochází k vlastní syntéze nových řetězců. Celý cyklus je obvykle opakován, dokud nedojde k syntéze dostatečného počtu kopií. Namnoženou DNA je pak možné vizualizovat v agarózovém elektroforetickém gelu pomocí UV záření a vhodného barviva (Birt & Baker 2009). Díky schopnosti amplifikovat i malé množství DNA jsou detekovatelné i mikroorganismy vyskytující se v přírodě v menšinovém zastoupení, což umožňuje také studium mikro habitatů (Wintzingerode et al. 1997). Ačkoliv je to rutinní metoda pro identifikaci čistých kultur, u environmentálních vzorků může dojít k inhibici PCR vlivem kontaminantů, diferenciální amplifikaci nebo tvorbě artefaktů PCR produktů (Wintzingerode et al. 1997).

V mikrobiální ekologii je pro kvantifikaci a expresi taxonomických a funkčních genů široce využívaná metoda kvantitativní PCR (Q-PCR nebo také PCR v reálném čase). Analýza Q-PCR kombinuje polymerázovou řetězovou reakci s fluorescenční detekcí. Prostřednictvím selektivně navázaného chromoforu zaznamenává hromadění amplikonů v „reálném čase“ během každého cyklu amplifikace, což umožňuje kvantifikaci genů. Pokud Q-PCR předchází reverzní transkripce, může být stanovena i míra genové exprese (RT-Q-PCR) (Smith & Osborn 2009). Velmi rozšířené jsou takzvané „fingerprintové metody“. Na základě porovnání genetických profilů lze hodnotit diverzitu komunit, ale i vnitrodruhové variace na úrovni jednotlivých kmenů. Náhodná amplifikace polymorfní DNA (RAPD) je v molekulární ekologii běžná metoda pro identifikaci, hodnocení příbuzenských vztahů, analýzu směsných vzorků, nebo pro tvorbu specifických sond. Od klasické PCR se liší v tom, že pro amplifikaci je použit pouze jeden oligonukleotidový primer o libovolné sekvenci, který náhodně přisedá na více míst DNA a vznikají tak fragmenty o různé délce. Produkty amplifikace jsou vizualizovatelné na

agarózovém gelu a polymorfismus slouží jako hlavní genetický marker (Hadrys et al. 1992, Franklin et al. 1999). Obdobnou metodou je interrepetitivní polymerázová řetězová reakce (REP-PCR). Pro amplifikaci je také použit pouze jeden primer, ovšem komplementární s repetitivními sekvencemi uvnitř genomu (Ward & Roy 2005). Polymorfismu délky restrikčních fragmentů (RFLP), známý také jako restrikční analýza amplifikované ribozomální DNA (ARDRA) je dalším nástrojem ke studiu mikrobiální diverzity. PCR amplifikované fragmenty DNA jsou štěpeny restrikčními enzymy. Různě dlouhé fragmenty jsou opět detekovány na agarózovém nebo polyakrylamidovém elektroforetickém gelu. Tato metoda může být použita buď pro studium diverzity, pro odlišení standardního genu od zmutovaného, nebo studium klonů (Kirk et al. 2004). Modifikací této metody je polymorfismus délky terminálních restrikčních fragmentů (T-RFLP). V tomto případě jsou PCR produkty fluorescenčně označeny a následně štěpeny endonukleázou. Označit lze jen jeden nebo oba primery použité pro PCR reakci. V případě, že jsou značeny oba primery, lze každý označit jiným fluorescenčním barvivem. Výsledné produkty jsou porovnány s rozdílně značeným hmotnostním standardem na gelové nebo kapilární elektroforéze. Detekovány jsou jen koncové (terminální) restrikční fragmenty. Výsledkem je zjednodušený fingerprint mikrobiálního společenstva ve formě elektroferogramu, který znázorňuje profil komunity, jako řadu píků různých výšek (Osborn et al. 2000). Popsány jsou i další metody založené na PCR, jsou to například analýza polymorfismu délky intergenového spaceru mezi geny malé a velké ribozomální podjednotky (RISA), polymorfismus konformace jednořetězcových nukleových kyselin (SSCP), nebo gelová elektroforéza v denaturačním/teplotním gradientu (D/TGGE) (Kirk et al. 2004).

2.7.3.4. Sekvenování DNA

Cílem genomového sekvenování je stanovení primární struktury DNA, což umožňuje studium molekulárních procesů celého buněčného systému. Genomové sekvenování je také základem -omických technologií jako je proteomika nebo transkriptomika (microarray), a proto bývá označováno za vrchol revoluce v biologických vědách (Hall 2007). Existují dvě základní, principiálně odlišné metody. V obou případech jsou výchozím materiálem pro sekvenaci fragmenty DNA s přesně definovanými konci, které jsou nejčastěji získávány klonováním do vhodného vektoru nebo PCR, a označené na 5´ konci radioaktivním izotopem ^{32}P. Společným rysem je i snaha vytvořit soubor fragmentů nukleových kyselin, které se vzájemně liší délkou o jeden nukleotid. První z nich, Maxamovo-Gilbertovo sekvenování, označované také jako

metoda chemická, je založeno na degradaci řetězců pomocí chemických sloučenin v místech, kde je lokalizovaná báze určitého typu. Cílené fragmenty jsou rozdělené do 4 vzorků a do každého přidány příslušné chemikálie, které naruší vazbu mezi nukleotidem a cukernou složkou. Reakční podmínky a činidla jsou zvolena tak, že dochází k modifikaci G, A + G, C + T, nebo C, a to průměrně 1 báze v řetězci. V dalším kroku jsou vysokou teplotou a chemickými činidly vazby v místě modifikace štěpeny, takže vznikají různě velké fragmenty. Vzorky z každé z reakčních směsí jsou naneseny na gel, elektroforeticky separovány a fragmenty se značenými konci vizualizovány pomocí radiografie. Na základě velikosti fragmentů je pak určována posloupnost jednotlivých nukleotidů v řetězci (Maxam & Gilbert 1977). Prakticky souběžně vznikla i druhá metoda, Sangerova. Ta bývá označována za metodu enzymatickou. Na rozdíl od chemické, není založena na degradaci, ale naopak na syntéze komplementárních vláken o různé délce. Do reakční směsi pro amplifikaci jsou přidány kromě normálních nukleotidů i jejich 2',3'-dideoxy analogy (ddNTP), které postrádají 3'-OH skupinu. Tyto analogy fungují jako koncové inhibitory (terminátory). V důsledku chybějící 3'-OH skupiny nemůže DNA polymeráza připojit další nukleotid, a tak vznikají různě dlouhá vlákna v závislosti na pozici jejich začlenění do řetězce. Stejně jako u chemické metody, je sekvenování prováděno ve 4 oddělených vzorcích, které obsahují jeden ze 4 analogů ddNTP. Po procesu amplifikace se značené fragmenty opět separují, vizualizují a vyhodnocují na základě retenčního času (Sanger & Nicklen 1977). V originálním protokolu byly cílené DNA fragmenty značeny radioaktivními izotopy, ty ale byly záhy nahrazeny fluorescenčními barvivy, což mimo jiné umožnilo amplifikaci a následnou separaci jediného vzorku (Prober et al. 1987). Díky výhodám, které plynou především z eliminace práce s toxickými látkami a radioizotopy, se Sangerova metoda stala na 30 let jedinou rutinně využívanou sekvenační technikou (Schuster 2008). Jednou z novějších technologií a nástupcem enzymatické metody je pyrosekvenování. Tato metoda je založena na detekci pyrofosfátu (PPI) uvolňovaného v průběhu syntézy DNA. V kaskádě enzymatických reakcí se vytváří viditelné světlo, které je úměrné počtu zabudovaných nukleotidů (Ronaghi 2001). Od roku 2005, kdy vznikla sekvenační technologie 454, pak hovoříme o tzv. Next generation sequencing (NGS). Jedná se o vysoce výkonné technologie, které se v mikrobiologii uplatňují zejména při analýze celých metagenomů a plně nahradilo, dnes již nepoužívané, pyrosekvenování. Na buněčné úrovni NGS umožňují, jako první technologie, studium všech mutací v genomu organismu. Toho se využívá například při identifikaci alel, které jsou zapojeny do vzniku antibiotické rezistence (Schuster 2008).

3. Hypotéza

Je známo, že mořské druhy mechovek osidluje variabilní mikrobiota, která produkcí sekundárních metabolitů ovlivňuje okolní mikro- a makroorganismy. Jednou z hlavních biologických funkcí sekundárních metabolitů mechovek a s nimi souvisejících mikroorganismů je antimikrobiální aktivita. Předpokládáme, že charakterizace bakterií kolonizujících *P. magnifica* pomůže přispět k objasnění možného vliv této mechovky na původní ekosystém, a že některé z izolovaných bakterií budou mít schopnost produkovat antimikrobiálně působící látky. Takové látky lze použít například pro farmaceutické účely, pro modulaci trávicího traktu různých druhů živočichů nebo jako konzervační činidlo.

4. Cíl práce

Cílem doktorské dizertační práce je izolovat a identifikovat bakterie sladkovodní mechovky *Pectinatella magnifica*. Dále je cílem testování antimikrobiální aktivity těchto bakterií a extraktů připravených přímo z kolonií *P. magnifica*, proti potenciálně patogenním mikroorganismům, a psychrofilním bakteriím způsobujícím kažení potravin.

5. Publikované práce

5.1. Methods of studying diversity of bacterial communities: A review

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METHODS OF STUDYING DIVERSITY OF BACTERIAL COMMUNITIES: A REVIEW*

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Bacterial life is a predominant component of all environments, occurring in communities usually of complex diversity. Bacteria are engines of globally important processes which makes them subject of interest to many scientific studies. Although many kinds of methods have been developed and microbial ecology has undergone a profound change in the last two decades with regard to methods employed, the analysis of community composition and function still remains a great challenge. In this article we present an overview of methods commonly used for the study of bacterial diversity. Emphasis was placed on cultivation, biochemical and chemotaxonomic, and molecular-genetic based methods.

bacterial diversity, community composition, cultivation, biochemical methods, chemotaxonomy, molecular-genetic methods

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INTRODUCTION

Bacteria belonging to prokaryotes are ubiquitous and essential components of the Earth's biota. They catalyse unique and indispensable transformations in the biochemical cycles of biosphere, produce important components of Earth's atmosphere, and represent a large portion of life's genetic diversity. They are also producers of important compounds that serve as a nutrient source, usable by all parts of the food chain. The numbers of prokaryotes on the Earth are estimated to be 4–6 × 10^{30} cells. Most of them occur in the open ocean, in soil, and in ocean and terrestrial subsurface (W h i t m a n et al., 1998). Another very important microbial ecological niche is animal body. It has been estimated that the microbes in human bodies collectively make up to 100 trillion cells, 10-fold the number of human cells, and suggested that they encode 100-fold more unique genes that our own genome. The majority of these microbes reside in the gut, have a profound influence on human physiology and nutrition, and are crucial for human life (Q i n et al., 2010). Because of the immense importance of bacteria, there is a need for more detailed and predictive understanding of the bacterial communities responsible for activities mentioned above and how they may respond to environmental stress and changes (H i r s c h et al., 2010).

The late 19th century is considered as the beginning of bacterial taxonomy and Ferdinand Cohn in 1872 was the first who classified six genera of bacteria mainly based on their morphology. At that time

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growth requirements and pathogenic potential were the most important taxonomic markers, besides morphology. Many of the pathogenic bacteria known today were discovered just between 1880 and 1900. At the beginning of the 20th century physiological and biochemical features started to be used, in addition to morphology, as important markers for classification and identification of microorganisms. Later enzymes have been studied and metabolic pathways have been described. Since the 60s of the 20th century microorganisms have been also identified and classified based on chemotaxonomy (composition of cell constituents) and genotype (Schleifer, 2009). In the 80s of the 20th century scientists have found the discrepancy between counts of cells visible under the microscope and the equivalent total viable counts after the cultivation. This phenomenon, so-called 'great plate count anomaly', was the first indication that majority of bacteria and archaea on Earth remain 'unculturable'or 'as yet uncultivated' on artificial media *in vitro*. This fact has contributed to the development of molecular biological methods to study microbial communities, without the need of cultivation (Vartoukian et al., 2010; Uhlík et al., 2013). In nature less than 1% of bacteria can be cultured with currently available methods. Relative proportion of bacteria growing on agar plates (colony forming units – CFU, determined as a percentage of culturable bacteria in comparison with total cell amount) vary from 0.1 to 1% in pristine forest soils and to 10% in environments like arable soils (Torsvik et al., 1998; Cottrell, Kirchman, 2000). Amann et al. (1995) reported culturability of bacteria in water 0.001–3%, activated sludge 1–15%, sediments 0.25%, and in soil 0.3%. Studies performed in the 1970s using anaerobic culture-based techniques identified more than 400–500 distinct bacterial species in the human gut, but approximately 60–80% of gut microbes simply cannot be grown by conventional *in vitro* techniques (Dave et al., 2012). Despite the fact that cultivated microorganisms represent only a minor part of microbial diversity in nature, cultivation is essential for isolating live bacterial cells in pure culture and determining their metabolic pathways. Therefore cultivation is still one of the key techniques used in microbiology (Uhlík et al., 2013).

Most of information on bacterial diversity concerns genetic and taxonomic diversity, but to understand the role of communities in different environments it is essential to have knowledge of both community and functional diversity. Therefore, there are various approaches to study the microorganisms at the community level. In principal, these methods can be divided according to different criteria. In this article we divided them into cultivation, biochemical and chemotaxonomic, and molecular-based methods. Another criterion for grading methods can be for example taxonomic level because each technique is suitable for identification at various taxonomic levels.

Cultivation methods

Cultivation methods are based on inoculation and incubation of growth media and have been used for more than 100 years to detect microorganisms. These techniques provide at least two benefits: amplification of microbial material and purification of single organisms along with their direct descendants, which allows their further characterization. The composition of culture media depends on the aims of study. Elective media with rich sources of nutrients are used to obtain the widest possible spectrum of bacteria from environmental samples (Mandal et al., 2011). On the contrary, for enumerating and isolating a certain group of microorganisms media consisting of specific nutrients composition, energy sources and selective antimicrobial agents are required (Vlková et al., 2015). Since 1990 a range of chromogenic media have been developed that are designed to target microorganisms with high specificity, without the need of subsequent identification. Such media exploit enzyme substrates that release coloured dyes upon hydrolysis, thus resulting in target microorganisms forming coloured colonies that can easily be differentiated from other bacteria species. Ideally, other bacteria should either be inhibited completely by selective agents or form colourless colonies thus allowing target bacteria to 'stand out' against background flora (Perry, Freydière, 2007). Currently conventional bacterial testing methods relying on selective and chromogenic media are the most commonly used for detection and identification of indicator and pathogenic microorganisms in food and clinical microbiology (Relman, 1998; Mandal et al., 2011). Serial enrichment of microorganisms in the presence of various nutrient and energy sources, analyses of their macromolecular composition and their metabolic by-products, and the use of specific immunologic reagents have created a variety of systems for microbial classification and identification (Relman, 1998). These methods are very sensitive, inexpensive, except chromogenic media, which are invariability more expensive than conventional media and can give both qualitative and quantitative information on the number and the nature of microorganisms present in a sample. On the other hand, these methods are time consuming. They require several days to give results, because they rely on the ability of the organisms to multiply to visible colonies. Moreover, culture medium preparation, inoculation of plates, and colony counting makes these methods labour intensive. Limitations of cultivation methods may be due to heterogeneity of samples matrices, physical form or different viscosity owing to the content of fats and oils. Moreover, bacteria are not uniformly distributed in environment or in samples. Another limitations include the difficulty in dislodging bacteria or spores from soil particles or biofilms, incomplete selectivity of culture medium, growth conditions, and different

Table 1. Advantages and limitations of the cultivation method

Method	Advantages	Limitations
Cultivation	sensitive	'unculturable' and 'as yet uncultivated' bacteria not detected
	inexpensive	dislodging bacteria from sample
	amplifies microbial material	selectivity of culture medium
	qualitative and quantitative information	different growth rate
		slow

growth rate of microorganisms (Kirk et al., 2004; Mandal et al., 2011), see Table 1.

Biochemical and chemotaxonomic methods

Sole carbon source utilization profiles. The principal of this technique is based on the ability of bacteria to utilize different substrates under various conditions. Microorganisms are cultivated in microtiter plates containing different carbon sources and indicator in wells. In the case that substrate is metabolized the degradation is accompanied by colour change. Methods of sole carbon utilization profile are generally used for characterization (detection of metabolic pathways and enzymes) and strain identification of pure cultures, but also allow studying functional aspects at community level. The first aspect is microbial 'community function', which implies actual expressed catabolic activity. In contrast, 'functional diversity' indicates its potential activity, i.e. the capability of the community to adapt metabolism (catabolism) and/or the relative composition and size of constituent populations to varying abiotic conditions (microclimate and added substrates). Information on both functional aspects is essential (Preston-Mafham et al., 2002). Currently, various types of biochemical kits are commercially available. For most of them the inoculation of pure culture into wells is required. Systems that may determine metabolic potential of whole microbial communities in the studied environment are also available (Stefanowicz, 2006).

The first who studied microbial communities by biochemical systems (on the basis of patterns of community-level sole-carbon-source utilization) were Garland and Mills (Garland, Mills, 1991). They used commercially available microplates developed by BIOLOG, Inc., designed for bacterial isolates identification, which allow simultaneous testing of 95 separate carbon sources. Portions of whole environmental samples instead of a single organism were inoculated into the wells. Utilization of substrates by bacteria was determined by the colour change of tetrazolium violet after the reduction by NADH produced by the microorganism during the reaction. The intensities of colour changes were determined spectrophotometrically. The substrate utilization rate between different groups of microorganisms is varied, therefore high variability in velocity and density of colour development can be observed, depending on microbial community composition. The microbial communities are consequently characterized by their metabolic fingerprints. Currently, special plates designed for ecological study of whole microbial communities are produced, e.g. Eco plates (Biolog, Inc.) containing 31 different substrates in triplicate, which allows statistical analysis (Baudoin et al., 2001; Stefanowitz, 2006; Button et al., 2015). This method is applied especially for evaluation of relative differences between functional diversity (the role of individual components) of microorganisms, for example in plant rhizospheres or communities in sites contaminated by industrial pollution, it does not allow identification of species composition in a sample. Limitations of metabolic profiling are that the method selects microorganisms capable of growing under the experimental conditions, favours fast growing microorganisms, is sensitive to inoculum density, and reflects the potential, and not *in situ*, metabolic diversity. Moreover, the carbon sources used for the tests may not correspond to those present in the sample (Kirk et al., 2004), see Table 2.

Fatty acid analysis. Fatty acids, integral part of membrane lipids, are essential components of all living cells. They have a greatly diversified structure and simultaneously are highly biospecific, therefore are used as biomarkers for studying microorganisms. Microbial fatty acid analysis may serve for both identification and taxonomic classification of pure cultures, and studying microorganisms in environmental samples or laboratory cultivated bacterial mixtures (Zelles, 1999).

A technique widely used for bacteria identification is the fatty acid methyl ester analysis (FAME), which is based on a four-step procedure: extraction of fatty acids from cellular lipids, methylation of fatty acids, extraction of FAMEs, and their analyzation by gas chromatography (GC). Cellular lipids are present in viable or non-viable cells, so extraction of fatty acids is not restricted to living organisms. In environmental studies the fact that in complex samples both cellular and extracellular lipids are found must be taken into consideration. Extracellular lipids may exist in a stable form in the sample organic matter, so results do not provide information about actual diversity but rather insight into the history. In addition, extracellular lipids

Table 2. Advantages and limitations of biochemical and chemotaxonomic methods

Method	Advantages	Limitations
Sole carbon source utilization profiles	fast relatively inexpensive differentiates between communities	selects culturable fraction of community favours fast growing organisms sensitive for inoculum density reflects the potential, not *in situ* metabolic diversity tested carbon sources may not correspond to those present in sample does not allow identification of species composition
Fatty acid analysis	culture independent detects viable cells, or both live and dead qualitative and quantitative information sensitive to changes in community structure identifies specific organisms	identification restricted to culturable bacteria fatty acids derived from eukaryotic organisms not all species can be detected not all species can be distinguished from complex profile
Protein analysis	generates large amount of data informs about gene functions identifies specific organisms links functions with identity detects changes under varying conditions informs on post-translational modification	technologically challenging demanding

can be derived not only from bacteria but also from eukaryotic microorganisms and macroorganisms, e.g. plants (Haack et al., 1994; Zelles, 1999).

A similar approach is the analysis of the phospholipid fatty acid (PLFA) pattern of bacteria. In the first phase all cellular lipids from a sample are extracted by suitable solvents and buffer, and subsequently divided into fractions: neutral lipids, glycolipids, and phospholipids (polar lipids). Methyl esters of phospholipid fatty acids, prepared by mild alkaline methanolysis, are analysed by gas chromatography or mass spectrometry (GC/MS). Phospholipids are an essential constituent of all cellular membranes and are used as a marker for microbial community description because their derived fatty acids significantly differ between individual bacterial groups. Whereas phospholipids rapidly degrade after cell death and do not occur in storage components, the method provides information about the actual microbial composition. The FAME analysis, in comparison with PLFA, is more rapid and less demanding than PLFA, on the other hand provides less reliable results (Makula, 1978; Gattinger et al., 2003). Both methods mentioned above are suitable for studying bacteria and eukaryotic microorganisms, in which fatty acids are connected by ester bonds. Archeal polar lipids contain fatty acids connected by ether bonds, thus they are not hydrolysed using the standard PLFA protocol. Polar lipids in Archea (so-called phospholipid ether lipids) have a unique structure, and can be easily distinguished from bacterial and eukaryotic phospholipids. They are composed of di- and tetraethers of glycerol or more complex polyols with side chains consisting of isoprenoids, which can only be liberated after ether cleavage with a strong acid such as hydriodic acid (Gattinger et al., 2003).

A method based on cellular lipids analysis is also the analysis of isoprenoid quinones, which represent an important group of isoprenoid lipids occurring in the cytoplasmic membrane of most prokaryotes. The isoprenoid quinones analysis is based on quinones extraction by organic (nonpolar) solvents, purification, and analyzation using various chromatographic methods, UV spectrophotometry, or mass spectrometry. This method is applicable to all environmental samples from which an absolute amount of microbial biomass $\geq 10^9$ cells can be collected, and in combination with molecular methods it should provide accurate and reliable information about population dynamics and community structure (Collins, Jones, 1981; Hiraishi, 1999; Schleifer, 2009). Isoprenoid quinones play an important role in electron transport, oxidative phosphorylation and, possibly, active transport, and thus they constitute a part of both respiratory and photosynthetic system of microorganisms. Two major structural groups of bacterial isoprenoid quinones can be recognized: naphthoquinones and benzoquinones. For classification not only the type of quinone, but also length and saturation of polyprenyl side chain is significant.

In addition, for microbial community description other fatty acid analysis based methods have been used, including the analysis of muramic acid, teichoic acid components, or lipopolysaccharides fatty acids charac-

teristic for Gram-negative bacteria and cyanobacteria (Gehron et al., 1984; Vestal, White, 1989).

Fatty acid profiles of pure cultures are compared to each other or to an established database to assist in the polyphasic taxonomic description of these organisms. The community structure is interpreted based on the database of pure cultures peripherally, unless there is a unique lipid which can serve as a true biomarker for given microbial strains (Zelles, 1999; Hopkins et al., 2001). Information about the community composition is based on the frequency and ratio between individual fatty acids, which form relatively constant portion of cell biomass. So, changes in fatty acids profiles represent changes in microbial population (Kirk et al., 2004). Even though fatty acids analysis represents a culture independent technique, determination of the community composition by software is restricted only to reference fatty acid profiles from individual organisms grown on the culture medium. Further, not all bacterial species can be detected. Disadvantageous is also the fact that some bacterial species are not sufficiently characteristic in respect to fatty acids composition, and thus cannot be distinguished from the whole community profile (Haack et al., 1994; Zelles, 1999).

Protein analysis. Another way how to study bacteria is to examine the protein content. The proteins found in bacteria provide not only an indirect genetic information on the organism, but are also found in a great abundance, which in turn should lead to a high detection sensitivity. The type of proteins present in bacterial cells is immense and their amounts vary greatly. Some proteins are unique to specific bacteria and may serve as biomarkers. The ribosome is an organelle found in all cells that coordinate protein synthesis, therefore ribosomal proteins have been proposed as suitable biomarkers for chemotaxonomic characterization of bacteria (Teramoto et al., 2007).

Complex information about the protein composition provides the field of proteomics, which is traditionally defined as the analysis of a complete set of proteins (proteome) of a given cell or organisms at a given time under specific conditions. The term metaproteome reflects the compound proteome of whole microbiota found in the environment, i.e. mixed microbial communities (Wilmes, Bond, 2004). Metaproteomics is widely accepted as a key technique in the postgenomic era to investigate global protein synthesis and gene expression. In this context, the large-scale study of proteins expressed by indigenous microbial communities should provide information to gain insight into the functional dimension of the environmental genomic dataset and help achieve a major goal of environmental microbiology: the ability to link individual bacterial species to function (Cash, 2000; Wilmes, Bond, 2006). More specifically, analyses of metaproteome allow tracking new functional genes and metabolic pathways, and identifying proteins preferentially associated with specific stress (Maron et al., 2007). The metaproteome analysis implies the development of different technical steps, from the extraction of proteins from the environmental matrix to the resolution of their diversity and identification. For an exhaustive recovery of proteins (cellular + extracellular), bacteria may be lysed either directly in the environmental matrix before extraction, purification, quantification, and analysis or by an indirect strategy, in which proteins are extracted, purified, and separated from organisms that have been previously extracted from the environmental matrix. The most crucial step in metaproteomics is the protein pool extraction. The complexity of indigenous microbial communities, the heterogeneity of natural environments, and the presence of interfering compounds make the extraction difficult (Wilmes, Bond, 2006). Once the protein samples are obtained, different biochemical methods can be applied for metaproteome analyses according to the type of information and the level of required results. In general, the combination of separation techniques (electrophoresis, chromatography) and proteins identification by mass spectrometry (MS) provides the best possible results. The traditional way to separate proteins in a complex mixture is by the use of two-dimensional (2D) gel electrophoresis, when proteins are separated according to net charge in the first dimension (isoelectric focusing, IEF) and by molecular weight in the second dimension (SDS-PAGE). The 2D electrophoresis has the ability to resolve a large number of proteins including those with post-translational modification as well as unique forms of proteins that result from differential mRNA splicing or proteolysis and provide 'proteofingerprint", which can be analysed using computer software to identify changes in protein expression in the two samples. Subsequently spots of interest may be excised, digested by trypsin, and analysed. However chromatographic based techniques are often preferred as a more reliable tool for proteins separation (Wilmes, Bond, 2004; Maron et al., 2007). Although various types of mass analysers are used in microbial proteomics including tandem mass spectrometry (MS-MS), quadrupole mass spectrometers or ion trap mass spectrometers, a revolutionary progress in protein analysis has occurred with the onset of two critical 'soft ionization' technologies, namely electrospray ionization (ESI) MS and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS (Graham et al., 2007). Especially MALDI-TOF MS is widely used not only in environmental analyses but for routine classification and identification of bacteria, based on characterization of ribosomal subunit proteins as biomarkers, in numerous fields, including the food industry and public health (Teramoto et al., 2007).

Proteomics provides qualitative data on the proteins encoded by the bacterial genomes together with quantitative data on the response of proteins synthesis

Table 3. Advantages and limitations of molecular-based methods

Method	Advantages	Limitations
Labelling	studies metabolically active fraction links functions with identity DNA, RNA, PLFAs or proteins can be targeted studies *in situ* sensitive	expensive labelled substrate sensitivity of DNA stable isotope probing interpretation of data
Nucleic acid hybridization and re-association	phylogenetic identification studies *in situ* DNA or RNA can be studied thousands of genes can be analyzed qualitative and quantitative information	technically demanding error prone comparative database cannot be built less sensitive not accurate in samples of complex diversity design of specific probes
Polymerase chain reaction (PCR)-based methods	amplifies specific RNA fragments *in vitro* sensitive phylogenetic identification qualitative and quantitative information studies gene expression	inhibition by co-extracted contaminants differential amplification formation of artefactual products formation of chimeric molecules formation of deletion and point mutants choice of primers

under defined environmental condition (Cash, 2000). Using this approach to elucidate functional components of microbial ecosystems has huge potential for the study of environmental microbiology but still poses enormous challenges (Wilmes, Bond, 2006).

Molecular-genetic methods

The basic premise of molecular-genetic methods is that you are working on nucleic acids. DNA extracted from a sample represents the total metagenome, including components that are no longer viable, whereas RNA is synthesized only by active growing cells and degrades relatively rapidly. In prokaryotes, messenger RNA (mRNA) is usually very short-lived and indicates which genes are active at the time of extraction, but ribosomal RNA (rRNA) is more stable as it possesses secondary structure and is associated with ribosomal proteins, so in theory, it could survive for months in moribund or dead cells in environmental sample. However, in cells that are or recently have been active, there are many thousands of molecules of rRNA. Thus the analysis of rRNA abundance and diversity has been used to indicate the most active bacterial population, despite the fact that the number of ribosomes varies between groups. More precise information relevant to particular functions can be obtained from mRNA but it presents more technical difficulties. Both mRNA and rRNA can be converted to DNA using enzyme reverse transcriptase (RT) (Hirsch et al., 2010). The best characterized molecules used for bacterial systematics are small subunit rRNA gene sequences (16S rRNA). These molecules are universally distributed in the cells, exhibit constancy of function, change in sequence very slowly, the number of mutations corresponds to the evolutionary distance between strains, moreover contain high conservative regions across the bacteria domain and simultaneously variable regions specific for a certain taxon. Additional advantage of 16S rRNA is that it could be easily isolated (Fox et al., 1980; Uhlík et al., 2013). The analysis of rRNA genes provides a framework for assigning sequences to genera and species, appropriate for investigating the microbial community diversity, but it cannot always resolve the species and provides insufficient discrimination at the sub-species level. For intra-species variation, protein-encoding genes that have higher levels sequence variation will permit differentiation of closely related individuals. An added advantage is that most of these genes occur with only a single copy per genome, and therefore give a more reliable indication of the relative abundance of different groups than the 16S rDNA (Hirsch et al., 2010).

While microbiologists were previously limited by their inability to characterize uncultured organisms, the advent to so-called 'cultivation independent' methods has provided researchers with the ability to determine the composition of bacterial communities and identify numerically important, but not yet cultured organisms (Forney et al., 2004). Molecular-genetic methods represent a culture-independent approach, which is characterized by high specificity and the detection limit is approximately 10^3 of cells/g or ml of sample, depending on a particular method (Mandal et al., 2011). They began to be applied since mid-1960s, when protein electrophoresis was firstly used in ecol-

ogy researches to detect genetic variation in samples of individuals from different populations and species (B a k e r, 2009). In microbiology these methods started to be used later, because techniques that were optimized for eukaryotic cells were not fully applicable for prokaryotes. Currently, molecular-genetic methods are commonly used for identification, determination of the phylogenetic position of unknown species, and also for studying the microbial community composition (W i n t z i n g e r o d e et al., 1997; Z h a n g et al., 2002). The advantages and limitations of selected techniques are listed in Table 3.

Labelling to link functions with identity. Stable isotope probing (SIP) can be considered as a transition between biochemical and molecular based methods that allow studying biochemical processes involving the participation of microorganisms in the natural environment without the need of their cultivation. SIP exploits physical properties of the atoms that constitute all cellular components, in particular the isotopes of carbon. Substrates labelled by stable "heavy" isotopes are added into the environmental sample, metabolized by a certain group of microorganisms, and incorporated into their cellular structures. For substrate probing stable isotopes (especially ^{15}N, ^{2}H, and most commonly ^{13}C) are used. Incorporating a labelled isotope into the cells considerably increases the differences of the density between heavy (labelled) and light (unlabelled) fractions, which can be separated from each other by equilibrium density-gradient centrifugation (ethidium bromide, cesium chloride, cesium trifluoroacetate). Even small differences in the isotope ratio can be measured by isotope-ratio mass spectrometry (IRMS) or by secondary ion mass spectrometry (SIMS). For identifying the bacteria involved in the labelled substrate degradation serve biomarkers such as DNA, RNA, PLFAs or proteins which can be analysed by a range of molecular and analytical techniques (R a d a j e w s k i et al., 2003; N e u f e l d et al., 2007a; L ü n s m a n n et al., 2016).

Phospholipid-derived fatty acid SIP (PLFA-SIP) is the method of choice when probing a population composed of relatively low cell number or growth rates and therefore incorporating minimal amount of labelled substrate. However utility of PLFA-SIP may be limited, because resolving profiles composed of multiple species can be problematic. PLFAs are analysed by a combination of gas chromatography and isotope ratio mass spectrometry (GC-c-IRMS) without prerequisite separation (T r e o n i s et al., 2004; N e u f e l d et al., 2007b). More accurate identification results are provided by nucleic acids-based SIPs. RNA-SIP enables the analysis of 16S ribosomal RNA (rRNA) genes and DNA-SIP enables analysis of both 16S rRNA genes and physiological genes from target organisms. RNA-SIP is more rapid, almost 10-fold, because DNA-SIP experiments require cell division in the presence of labelled substrate to achieve sufficient incorporation for separation of labelled DNA, and also offers higher sensitivity than DNA-SIP. On the other hand, a sufficient amount of high-quality microbial rRNA for RNA-SIP is not extractable from all environmental samples. The advantage of DNA-SIP is a relatively easy DNA extraction and stability compared with rRNA (R a d a j e w s k i et al., 2003; W i n d e r l et al., 2010).

The second method, which has been developed to identify metabolically active bacterial groups in a sample, is 5-bromo-2′-deoxyuridine (BrdU) probing. BrdU is a thymidine nucleotide analogue that can be incorporated into the DNA of dividing cells. DNA extracted from cells that incorporated BrdU can be isolated by immunocapture and then compared, using any method appropriate for community DNA analysis, to the unlabelled DNA from the less active majority (H i r s c h et al., 2010). Thanks to this method it is possible to determine the most active bacterial groups in environmental samples under various conditions. BrdU probing has been used to study microbial community changes, to detect microbes that grew in response to the availability of various carbon sources or in agricultural and bioremediation studies, both *in vitro* and *in vivo* (S c u p h a m, 2007).

Nucleic acid hybridization and re-association. One of the most common techniques used in systematic studies, for locating homologous DNA sequences and measuring their overall base pair differences respectively, is DNA–DNA hybridization (DDH). The general principle of DNA–DNA hybridization requires shearing the DNA into small fragments (600–800 base pairs), dissociation of hydrogen bonds by high temperature to obtain single-stranded DNA, and subsequently re-association by decreasing temperature. DNA fragments of assayed organism and reference organisms are mixed, denatured, and re-associated to form a heterodublex (B l e d s o e, S h e l d o n, 2009). Also total community DNA from environmental samples can be used to determine whether two samples share the same kind of organisms, regardless species composition knowledge (T h e r o n, C l o e t e, 2000). Genetic relatedness between the bacterial strains is determined by quantitative measurement of the mutually connected nucleotide bases of DNA fragments after re-annealing (E z a k i et al., 1989). The specific pairings are between A-T and G-C, and the overall pairing of the nucleic acid fragments is dependent upon similar linear arrangements of these bases along the DNA (R o s s e l l ó - M o r a, A m a n n, 2001). W a y n e et al. (1987) reported that the phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA–DNA relatedness and with difference in melting temperature 5°C or less (also called the thermal denaturation midpoint, T_m), both values must be considered. However this best-known recommendation is not a strict standard, also more stringent DNA–DNA hybridization values

have been used. Although DNA–DNA hybridization was one of the few universally applicable techniques available that could offer truly genome-wide comparisons between organisms, it has several important drawbacks. Because relatively large quantities of DNA (in comparison with PCR-based techniques) of high quality are required, the whole process of performing DDHs often becomes rather time-consuming, labour intensive, and technically demanding. This method is also error prone and cannot be used to incrementally build up a comparative database, in contrast to sequence information. Due to great diversity of microbial populations this technique is not suitable for the study of complex environmental samples (Rosselló-Mora, Amann, 2001; Goris et al., 2007).

The gross genetical structure and diversity in bacterial communities have been assessed by DNA melting-profiles and re-association analyses. In these analyses, total prokaryotic DNA (mixture of DNA from different bacterial types that are present in different proportions) is extracted from environmental samples, thermally denatured, and then re-associated. The re-association of single stranded (melted) DNA is measured as a decrease in absorbance using a spectrophotometer. The hybridization and re-association rates depend on the sequence homology. Thus, as the microbial community diversity increases, the rate of re-association of DNA extracts from the community decreases. Under defined conditions the molar concentration of nucleotides in single stranded DNA at the beginning of the re-association and time in seconds needed for 50% re-association ($C_0t_{1/2}$) is proportional to the DNA complexity (heterogeneity) and can be used as a diversity index. DNA re-association can provide useful information on the overall diversity and changes in the community structure. However, higher resolution and more accurate information about gross genetical structure are provided by PCR-based methods (Torsvik et al., 1998).

The easiest way to detect specific nucleic acid sequences is through direct hybridization of a probe to bacterial nucleic acids. The hybridization techniques relying on the specific binding of single stranded nucleic acid probes are an important tool in molecular bacterial ecology (Theron, Cloete, 2000). Oligonucleotides (less than 20 nucleotides) or polynucleotides (more than 50 nucleotides) serve as the probes, designed from the known sequences of various specificity from domain to species specific probes. Species specific probes complement the most variable regions, while more general probes target more conserved regions of the molecule (Ezaki et al., 1989; Theron, Cloete, 2000). Hybridization can be done on extracted DNA, RNA or *in situ*. Early applications of *in situ* nucleic acid hybridization relied on the use of isotopically labelled oligonucleotides that bound to the RNAs and following autoradiography organisms could be identified, but currently fluorescent probes are often preferred. The method, known as fluorescent *in situ* hybridization or FISH, has been used successfully to study the spatial distribution of bacteria in various environmental samples. FISH is a staining technique that allows phylogenetic identification of bacteria on a single cell level in mixed assemblages without prior cultivation and also allows determine the cell morphology of uncultured bacteria. The principle of this technique is hybridization of specific fluorescent dye labelled rRNA-targeted oligonucleotide probes to the target sides and detection of hybridized cells by epifluorescence and confocal laser microscopy, or by flow cytometry. FISH with polynucleotide DNA probes and FISH with oligonucleotide probes targeted to mRNA have also been described. This technique is commonly used for both quantitative and qualitative determination of bacteria and for the study of spatial and temporal dynamics of individual bacterial populations in their habitat. However, identification of microorganisms from environmental samples of unknown composition can be limited because the design of specific probes is based on the sequence database which is not complete for all bacteria (Theron, Cloete, 2000; Pernthaler et al., 2001).

Base-pairing of complementary sequences by hybridization is also the principle of the DNA microarray technique. This method was first described in 1995 and it has the ability to simultaneously display the expression of thousands of genes at a time. Specific binding of DNA allows a target DNA or RNA to hybridize to a specific complementary DNA (cDNA) probes immobilized on the surface of array. Each probe is made of thousands of cDNAs or oligonucleotides, each specific for a gene, DNA sequence, or RNA sequence of interest. DNA microarray has a wide range of applications. Except microbial population studies it is currently being applied also for example in quality control, clinical diagnostics, biomedical research of cancer, or drug discovery and development (Hadidi et al., 2004).

Polymerase chain reaction (PCR)-based methods. Research in the fields of population and evolutionary biology has been revolutionized by the introduction of polymerase chain reaction (PCR). Using this technology, the researcher can *in vitro* amplify specific DNA fragments in a virtually unlimited quantity (more than 10^9 copies after 30 cycles of DNA synthesis) (Hirsch, 2010). PCR involves enzymatic synthesis of a particular DNA (or sometimes RNA) sequence. The DNA region to be amplified is determined by the base sequences of a pair of oligonucleotide primers, which are complementary to binding sites situated on either side of the target sequence. According to the selection of primers, it is possible to detect either functional genes or sequences specific for a certain group of bacteria (e.g. genus or species specific primers). These primers binding sites reside on opposite strands of the template DNA so that primers have their 3′ hydroxyl

ends oriented towards each other. DNA polymerase-mediated extension of each annealed primer therefore proceeds in the direction of the other primer (Birt, Baker, 2009). PCR is based on the three simple steps required for any DNA synthesis. Each cycle begins with denaturation of template DNA by high temperature (usually 94 or 95°C) into single strands. The temperature is then lowered to permit annealing of the primers to the appropriate sequences of each original strand (45–60°C). Finally the temperature is elevated to achieve optimal polymerase activity (70–72°C), and extend the anneal primers as directed by the template strands. The amount of PCR products increases geometrically. PCR products can be visualized by ultraviolet (UV) transilluminator after electrophoresis through agarose gel staining by an appropriate dye, they appear as a bright band of appropriate size, and then can be readily characterized using any of numerous techniques (Birt, Baker, 2009).

Due to the power of the PCR to amplify a small amount of DNA, organisms occurring in small number in an environment are now detectable. Also, the sample volume required for analysis is significantly reduced and micro-habitats are now open for investigation. PCR amplification has become the method of choice for obtaining rRNA sequence data from microbial communities or pure cultures. Full length 16S rDNA can be amplified either directly or after reverse transcription of rRNA with a set of primers binding to conserved regions of 16S rDNA/rRNA. Although it is a routine method for pure cultures, several problems arise when the methods are applied to environmental communities including inhibition of PCR amplification by co-extracted contaminants, differential amplification or formation of artefactual PCR product (Wintzingerode et al., 1997).

In microbial ecology, quantitative PCR (Q-PCR or real-time PCR) is now widely applied to quantify the abundance and expression of taxonomic and functional gene markers within the environment. Q-PCR-based analyses combine 'traditional' end-point detection PCR with fluorescent detection technologies to record the accumulation of amplicons in 'real time' during each cycle of the PCR amplification. By detection of amplicons during the early exponential phase of the PCR, this enables the quantification of gene (or transcript) numbers when these are proportional to the starting template concentration. When Q-PCR is coupled with a preceding reverse transcription reaction, it can be used to quantify gene expression (RT-Q-PCR) (Smith, Osborn, 2009). The random amplified polymorphic DNA (RAPD) is a common PCR-based DNA fingerprinting technique used in molecular ecology to determine taxonomic identity, assess kinship relationships, analyse mixed genome samples, and create specific probes. The amplification protocol differs from the standard PCR conditions in that only a single random oligonucleotide primer is employed and no prior knowledge of the genome subjected to analysis is required. The amplification products are resolved on agarose gels and polymorphisms serve as dominant genetic markers, band patterns are compared to determine percent similarity. Main advantages of the RAPD technology include suitability for work on anonymous genomes, applicability to problems where only limited quantities of DNA are available, efficiency, and low expense (Hadrys et al., 1992; Franklin et al., 1999). Restriction fragment length polymorphism (RFLP), also known as amplified ribosomal DNA restriction analysis (ARDRA), is another tool used to study microbial diversity that relies on DNA polymorphisms. In this way PCR-amplified rDNA is digested by cutting restriction enzyme. Different fragment lengths are detected using agarose or non-denaturing polyacrylamide gel electrophoresis. RFLP banding patterns can be used to screen clones, detecting structural changes in microbial communities or to distinguish the standard from mutated genes (Kirk et al., 2004). Terminal restriction fragment length polymorphism (T-RFLP) is a technique that addresses some of limitation of RFLP. It is based on the restriction endonuclease digestion of fluorescently end-labelled PCR products. Either one or both primers used in the PCR can be labelled. If both, each can be labelled with a different fluorescent dye. Upon analysis, including separation by gel or capillary electrophoresis with laser detection of the labelled fragments using an automated analyser, only the terminal, end-labelled fragments are detected. So this technique permits an automated quantification of the fluorescence signal intensities of individual terminal restriction fragments (T-RFs) in a given community fingerprint pattern (Osborn et al., 2000).

Besides the above mentioned techniques, there exist many other polymerase chain reaction-based techniques to estimate the microbial diversity and community composition, e.g. ribosomal intergenic spacer analysis (RISA) (Fisher, Triplet, 1999), single strand conformation polymorphism (SSCP) of DNA (Loisel et al., 2006), denaturing gradient gel electrophoresis (DGGE), and temperature gradient gel electrophoresis (TGGE) (Muyzer, Smalla, 1998).

CONCLUSION

There are various approaches to determinate the composition of microbial communities and their role in different environments. In addition to the classical tools mentioned in this article, modern methods such as next generation sequencing (NGS) have recently started to be applied. However due to its comprehensive character this field should be a subject of a separate review. Each of the commonly used techniques has its advantages and limitations. The selection of a suitable method depends especially on the aims of the

scientific research. However, combination of multiple techniques provides the best possible knowledge of the community diversity, structure, and function.

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5.2. Identification of microbiota associated with *Pectinatella magnifica* in South Bohemia

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Identification of microbiota associated with *Pectinatella magnifica* in South Bohemia

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Abstract: The bacterial diversity of *Pectinatella magnifica* colonies sampled from pounds in South Bohemia during the summer of 2012 was investigated. The bacterial counts determined after cultivation on modified yeast extract-tryptone agar (Oxoid) supplemented with glucose (1 g L^{-1}) varied from 4.22 to 6.61 and from 1.30 to 6.85 log CFU/g for aerobes and anaerobes, respectively. Higher counts were found in the superficial structures of *Pectinatella* colonies than in the inner gelled mass. Neither a trend in bacterial numbers at the individual site during the season, nor correlations between bacterial counts in *P. magnifica* and the surrounding water were observed. Fifty-four isolates were identified by sequencing the 16S rRNA gene and through MALDI-TOF MS analysis. Species of *Aeromonas* and *Aquitalea* were the predominantly isolated bacteria, but members of *Chryseobacterium*, *Herbaspirillum*, *Enterobacter*, *Lactococcus*, *Leuconostoc*, *Pseudomonas* and *Sphingomonas* were also found. As listed genera are wildly distributed in different water, soil, and plant samples, we conclude that*Pectinatella* colonies are inhabited by environmental bacteria. Nevertheless, a symbiotic relationship of these bacteria with *P. magnifica* cannot be excluded.

Key words: *Pectinatella magnifica*; bacteria; South Bohemia; isolation; cultivation; identification

Introduction

Pectinatella magnifica (Leidy, 1851), a freshwater organism belonging to the Bryozoa phylum, forms large colonies with zooids (individual organisms) on its surface. The inner part of colonies consists of a gelled mass, which is produced by these zooids. The gelled layer may achieve thicknesses ranging from a few millimetres to several tens centimetres (Opravilová 2006). In the ponds and water reservoirs of South Bohemia (the Czech Republic), *P. magnifica* is an invasive species that has expanded massively since 2003. The colonies can reach weights of up to 10 kg, but the average weight has varied between approximately 200 and 500 g, depending on the site and sampling date (Balounová et al. 2011). The colonies occur mostly in large numbers, and its life activity likely has significant influences on the food chain (water filtration). While there have been some studies focusing on the occurrence and population dynamics of *P. magnifica* (Joo et al. 1992; Rodriquez & Vergon 2002; Bernauer & Jansen 2006), information about the bacteria that associate with it and the possible impact of this association on the ecosystem is lacking. However, *Pectinatella* colonies have been viewed as an intermediate host for fish parasites. After microscopic and *16S rRNA* sequencing analyses, Cannig et al. (2002) established a new genus of microsporidia from *P. magnifica* called *Trichonosema* gen. nov. with one species, *Trichonosema pectinatellae* sp. nov.

Pukall et al. (2001) identified bacterial strains isolated from the marine bryozoan *Flustra foliacea* (L., 1758) (North Sea) by amplified ribosomal RNA restriction analyses and *16S rRNA* partial sequence analysis. *Shewanella frigidimarina, Pseudoalteromonas* ssp. and *Psychrobacter* ssp. were found as the predominant bacteria. Additional proteobacteria and Gram-positive bacteria were found. Alphaproteobacterial symbionts were described by molecular-genetic methods in the bryozoa *Watersipora* (Andersona & Haygood 2007). The bryozoans *Bugula neritina* (L., 1758) and *Bugula simplex* Hincks, 1886 harbour a bacterial symbiont characterised as a Gammaproteobacterium '*Candidatus* Endobugula sertula', which has been implicated as the source of the bryostatins tested for use in human cancer treatments. Long rod-shaped bacteria in *B. simplex* have been visualised by fluorescence in situ hybridis-

Table 1. Counts (log CFU/g) of culturable bacteria in *Pectinatella magnifica* colonies and surrounding water (mean ± standard deviation, $n = 3$) detected during the summer of 2012.

Site	Type of sample	July 10th		July 30th		August 15th	
		Aerobes	Anaerobes*	Aerobes	Anaerobes*	Aerobes	Anaerobes*
Cep	Gelled mass of *Petinatella* colony	4.22 ± 0.26	3.82 ± 0.62	4.90 ± 0.83	3.16 ± 0.46	4.79 ± 0.59	2.46 ± 0.21
	Superficies of *Petinatella* colony	4.98 ± 1.01	4.82 ± 0.84	5.42 ± 0.39	2.30 ± 0.51	6.21 ± 0.33	2.98 ± 0.53
	Water	3.76 ± 0.09	1.86 ± 0.18	2.19 ± 0.05	1.04 ± 0.12	4.60 ± 0.13	2.40 ± 0.05
Hejtman	Gelled mass of *Petinatella* colony	4.82 ± 0.85	3.29 ± 0.78	4.80 ± 0.45	3.76 ± 0.66	3.86 ± 0.62	1.30 ± 0.43
	Superficies of *Petinatella* colony	6.50 ± 1.13	3.70 ± 0.35	5.05 ± 0.49	3.84 ± 0.28	6.61 ± 0.84	3.56 ± 0.38
	Water	3.56 ± 0.03	1.96 ± 0.04	3.08 ± 0.05	1.90 ± 0.03	4.60 ± 0.09	1.26 ± 0.10
Kanclíř	Gelled mass of *Petinatella* colony	6.49 ± 0.87	5.85 ± 1.01	4.95 ± 0.63	4.30 ± 0.26	6.37 ± 0.77	6.85 ± 1.06
	Superficies of *Petinatella* colony	6.53 ± 0.91	6.52 ± 0.90	6.71 ± 0.85	5.27 ± 0.97	6.37 ± 0.82	4.82 ± 0.48
	Water	3.26 ± 0.16	1.74 ± 0.09	4.96 ± 0.14	2.08 ± 0.03	4.60 ± 0.09	2.10 ± 0.02
Veselí	Gelled mass of *Petinatella* colony	5.91 ± 0.53	3.26 ± 0.58	5.32 ± 0.21	3.87 ± 0.81	5.01 ± 0.64	3.78 ± 0.39
	Superficies of *Petinatella* colony	6.06 ± 0.18	4.15 ± 0.24	4.96 ± 0.62	3.81 ± 0.38	5.18 ± 0.92	3.67 ± 0.18
	Water	5.12 ± 0.12	2.90 ± 0.02	3.27 ± 0.13	1.64 ± 0.07	3.30 ± 0.02	2.06 ± 0.11

Explanations: *Includes facultative anaerobes.

ation and were identified by 16S rRNA-targeted PCR and sequencing as '*Candidatus* E. gebosa' (Lim & Haygood 2004). '*Candidatus* Endobugula' symbiont-derived compounds defend host larvae from predations (Lim-Fong et al. 2008).

Because marine bryozoan are harboured by symbiotic bacteria, there is a presumption that *P. magnifica* colonies may also serve as a host for bacterial species. Hence, the aim of this study was to isolate and identify bacteria from *P. magnifica* colonies collected in South Bohemia.

Material and methods

Sample collection

Samples of *P. magnifica* colonies and surrounding water were collected at four locations: Hejtman (48°57′ N, 14°56′ E), Kanclíř (48°57′ N, 14°53′ E), Cep (48°56′ N, 14°52′ E) and Veselí (49°10′ N, 14°42′ E) ponds. Three colonies from each location were collected at three different sampling times during the summer of 2012 (Table 1). The superficial layer of bryozoan colonies containing zooids was collected separately from the inner gelled mass. The superficial layer was aseptically scraped from the colonies by a scalpel, uncovered gelled mass was sterilised with ethanol and was cut out. *Pectinatella* samples for microbiota evaluation were aseptically transferred to vials containing oxygen-free peptone water, kept in a refrigerator and analysed within 5 hours after collection. Water samples were collected into a sterile vessel and kept cold at 4 ℃ until analysed.

Bacteria enumeration and isolation

Bacterial counts were determined by cultivation under both aerobic and anaerobic conditions. There are no recommended or well-established agars for the enumeration of bacterial symbionts of fresh water bryozoa. Therefore, two rich media described for marine bryozoan (González et al. 1996) with some modifications were used. Briefly, the first modification was yeast extract-tryptone agar (YT; Oxoid) supplemented with 1 g L^{-1} of glucose dissolved in water (yeast-tryptone-glucose-water agar; YTGW). Medium of the same composition was dissolved in *Pectinatella* extract instead of in the water (the second modification; yeast-tryptone-glucose-pectinatella agar; YTGP) to support the growth of the bryozoan symbionts. For the *Pectinatella* extract preparation, the whole homogenised colonies were boiled, filtered and sterilised. Cold extract was used for dissolving of medium components.

For the bacteria enumeration, *Pectinatella* samples were homogenised by a mixer and serially diluted in peptone water under anaerobic conditions. One mL of appropriate dilutions were transferred to sterile Petri dishes, which were immediately filled with YTGW or YTGP agar. Plates were incubated both in anaerobic jars (Anaerobic Plus System; Oxoid) at 25 ℃ for 5 days and under aerobic condition at the same temperature for 3 days. After incubation, all bacterial colonies were counted and data were expressed as a log CFU/g. Means and standard deviations were calculated from three replicates (bacterial numbers were determined in three *Pectinatella* colonies at each site). Five bacterial colonies representing a wide range of pigments and colony types were picked up from each cultivation variant of *Pectinatella* collected on the first sampling date (10th of July 2012). Bacteria were enriched in YTGW medium and identified. Bacteria in water samples were enumerated in triplicate using YTGW agar, cultivation was performed under the same conditions as described above.

Bacteria identification

A total of 135 isolates were evaluated for their purity and morphological characteristics using phase contrast microscopy and Gram staining. Fifty-four pure strains with different morphologies were used for detailed identification by sequencing and Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS).

Bacterial DNA was extracted using the PrepMan© Ultra Sample Preparation Reagent Protocol (Applied Biosystems). Regions carrying the *16S rRNA* (1490 bp) gene were amplified by PCR with the aid of primers fD1 and rP2 according to Weisburg et al. (1991). The three 500 bp long sequences representing the whole *16S rRNA* gene were then amplified using primers FP27/515R, FP3/RP16 and FP19/rP2 (Forster et al. 1995). The PCR was performed under the following conditions: 92 ℃ for 5 min; 35 cycles of 92 °C for 1 min, 52.5 ℃ for 90 s, and 72 °C for 2 min; 72 ℃ for 5 min. PCRs were carried out in 25 µl volumes containing 20 mM Tris/HCl, 50 mM KCl, 200 µM each deoxynucleoside triphosphate, 50 pmol each primer, 1.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Top-Bio, Czech Republic). Checked and purified DNA fragments were sequenced on a 3100-Avant

Genetic Analyzer (Applied Biosystems) with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Obtained raw sequencing data were checked and edited by Chromas Lite 2.1.1 programme available at website (http://chromas-lite.software.informer.com/2.1/). Sequence data were aligned with the CLUSTAL W package (Thompson et al. 1997) in BioEdit programme (Hall 1999) and the almost-complete *16S rRNA* gene sequences were compared with published sequences of related bacteria from the EMBL (EBI) and GenBank (NCBI) nucleotide databases using the BLAST program (Maidak et al. 1994).

The isolates were also identified by MALDI-TOF MS using the MALDI BioTyper (TM) system (Bruker Daltonik GmbH, Germany) according to Kmeť & Drugdová (2012). Fresh overnight cultures were centrifuged and bacteria were fixed in 70% ethanol and transported for the analysis. Samples were prepared according to the microorganism profiling ethanol-formic acid extraction procedure, as recommended by the manufacturer. Each sample spot was overlaid with 2 μl of matrix solution (saturated solution of α-cyano-4-hydoxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid), air dried and analysed. Bacteria were identified by an analysis of the obtained raw spectra using BioTyper software (version 2.0).

Results

Bacterial numbers

Counts of culturable bacteria in *Pectinatella magnifica* colonies and the surrounding water obtained during the summer of 2012 are listed in Table 1. Bacterial numbers after cultivation on YTGW agar did not significantly differ from those determined on YTGP medium, so only data from YTGW agar are shown. In most cases, bacteria were more numerous in the superficial structures of the bryozoan colonies. The number of aerobic bacteria varied from 4.95 ± 0.63 in the superficial matter collected from Kanclíř pond on the 30th of July to 6.61 ± 0.84 log CFU/g in the material from Hejtman pond on the 15th of August. More variable counts were obtained after anaerobic cultivation of the superficial structures of *Pectinatella* colonies. The lowest bacterial counts were observed in colonies collected from Cep pond (2.30 ± 0.51 log CFU/g) on the 15th of August and the highest counts were found in matter from Kanclíř pond (6.52 ± 0.90 log CFU/g) on the 10th of July. Similar to superficial structures of colonies, in the inner matter, higher bacterial counts were found after aerobic than anaerobic cultivation. The number of aerobic bacteria in the gelled mass varied from 4.22 ± 0.26 in samples collected from Cep pond on the 10th of July to 6.37 ± 0.77 log CFU/g in the material from Kanclíř pond on the 15th of August. Again, the bacterial numbers were more variable after anaerobic cultivation reaching counts from 1.30 ± 0.43 (Cep pond, the 15th of August) to 6.85 ± 1.06 (Kanclíř pond, the 15th of August) log CFU/g.

The bacterial numbers in water samples varied from 2.19 ± 0.05 to 5.12 ± 0.12 log CFU/g and from 1.04 ± 0.12 to 2.90 ± 0.02 log CFU/g after aerobic and anaerobic cultivation, respectively. With two exceptions, lower bacterial counts were found in water samples than in *P. magnifica*. Only water collected from Hejtman pond contained higher numbers of aerobes (4.60 ± 0.09 log CFU/g) than the inner gelled masses of the colonies (3.86 ± 0.62 log CFU/g) collected from the same site at the same time. Comparable numbers of aerobic bacteria were cultivated in water and gelled mass matter of *Pectinatella* from Kanclíř pond on the 30th of July (4.96 ± 0.14 and 4.95 ± 0.63 log CFU/g, respectively).

Bacteria identification

A total of 135 pure strains were isolated. Fifty-four isolates with different cell and colony morphologies were used for detailed identification by sequencing and MALDI-TOF MS analysis. The results of the bacteria identification are listed in Table 2. After a BLAST analysis of the full *16S rRNA* gene, all isolates were successfully identified and the similarity to their nearest phylogenetic neighbour was higher than 99.5%. Using the MALDI-TOF approach, identification was successful in 34 strains (63%). No reliable results were obtained in 20 cases (score value lower than 1.699, see Table 2). Three isolates were classified to the probable genus level (score value between 1.700 and 1.999), in 30 strains a secure genus and a probable species identification with score values 2.000–2.299 were observed, and only 1 strain reached the highest probability group (highly probable species identification) with a score higher than 2.300. A comparison of identification results based on sequencing and MALDI-TOF analysis (strains with score higher than 2.000) revealed 27 cases of the same species classification results being achieved. Four strains were identically identified only at the genus level. If the results obtained by both methods differed (or were not reliable based on MALDI-TOF), identification by sequencing was considered as more reliable. *Aeromonas veronii* was found to be the most abundant species (27 strains) in *Pectinatella* colonies, followed by *Aquitalea magnusonii* (9 strains), *Lactococcus lactis* ssp. *lactis* (4 strains), *Aquitalea denitrificans* (3 strains), *Enterobacter aerogenes* (2 strains), *Herbaspirillum lusitanum* (2 stains), *Herbaspirillum huttiense* (2 strains), *Sphingomonas pituitosa*, *Pseudomonas moraviensis*, *Leuconostoc pseudomesenteroides*, *Chryseobacterium gambrini*, and *Chryseobacterium culicis* (1 strain each).

Table 3 displays the abundance of the detected species in individual *Pectinatella* samples. *Ae. veronii* (18 strains) together with *Aq. magnusonii* (1 strain) were the only two species isolated from surface layers of *P. magnifica* colonies at three sampling sites (Kanclíř, Hejtman, Veselí). The bacterial composition at Cep was more diverse and half of the surface samples were classified as *Lactococcus lactis* ssp. *lactis* (4 strains). High diversity was also observed in the bacterial composition of the gelled mass of colonies from all examined locations. The most abundant species at two areas (Hejtman and Veselí) was *Aq. magnusonii* (7 out of 12 strains). *Ae. veronii* was the most numerous species (6 out of 10 isolates) in the gelled mass from Kanclíř pond. The highest bacterial diversity in the inner gelled

Table 2. The distribution (number of isolates and percentage) of bacterial species (as determined by *16S rRNA* sequence analysis) isolated from the superficial structure or gelled mass of *Pectinatella* colonies from four examined localities.

Strain code	*16S rRNA* based identification*	GenBank accession number	MALDI based identification	MALDI score value (reliability)
C_G_AE-1	*Sphingomonas pituitosa*	KJ186947	*Sphingomonas pituitosa*	1.698 (–)
C_G_AE-2	*Herbaspirillum lusitanum*	KJ186941	*Massilia timonae*	1.448 (–)
C_G_AE-3	*Aeromonas veronii*	KJ186898	*Aeromonas veronii*	2.146 (++)
C_G_AE-4	*Herbaspirillum huttiense*	KJ186943	*Herbaspirillum huttiense*	2.128 (++)
C_G_AN-1	*Leuconostoc pseudomeseteroides*	KJ186948	*Leuconostoc citreum*	2.173 (++)
C_S_AE-1	*Pseudomonas moraviensis*	KJ186948	*Pseudomonas koreensis*	1.993 (+)
C_S_AE-2	*Aquitalea magnusonii*	KJ186924	*Pseudomonas graminis*	1.427 (–)
C_S_AE-3	*Aeromonas veronii*	KJ186899	*Aeromonas veronii*	2.074 (++)
C_S_AE-4	*Chryseobacterium culicis*	KJ186950	*Chryseobacterium gleum*	1.619 (–)
C_S_AN-1	*Lactococcus lactis* ssp. *lactis*	KJ186937	*Lactococcus lactis* ssp. *lactis*	2.232 (++)
C_S_AN-2	*Lactococcus lactis* ssp. *lactis*	KJ186938	*Lactococcus lactis* ssp. *lactis*	2.344 (+++)
C_S_AN-3	*Lactococcus lactis* ssp. *lactis*	KJ186939	*Lactococcus lactis* ssp. *lactis*	2.270 (++)
C_S_AN-4	*Lactococcus lactis* ssp. *lactis*	KJ186940	*Lactococcus lactis* ssp. *lactis*	2.051 (++)
H_G_AE-1	*Aquitalea magnusonii*	KJ186926	*Pseudomonas abietaniphila*	1.424 (–)
H_G_AE-2	*Aeromonas veronii*	KJ186900	*Aeromonas ichthiosmia*	2.147 (++)
H_G_AE-3	*Aquitalea denitrificans*	KJ186936	*Pseudomonas azotoformans*	1.520 (–)
H_G_AE-4	*Chryseobacterium gambrini*	KJ186951	*Chryseobacterium* sp.	1.659 (–)
H_G_AN-1	*Aquitalea denitrificans*	KJ186934	*Pseudomonas syringae* ssp. *syringae*	1.284 (–)
H_G_AN-2	*Aquitalea magnusonii*	KJ186927	*Chryseobacterium gleum*	1.330 (–)
H_G_AN-3	*Aquitalea magnusonii*	KJ186928	*Gordonia rubripertincta*	1.419 (–)
H_G_AN-4	*Aquitalea magnusonii*	KJ186929	*Lactobacillus mucosae*	1.542 (–)
H_S_AE-1	*Aeromonas veronii*	KJ186901	*Aeromonas veronii*	2.037 (++)
H_S_AE-2	*Aeromonas veronii*	KJ186902	*Aeromonas veronii*	2.097 (++)
H_S_AE-3	*Aquitalea magnusonii*	KJ186930	*Clostridium cochlearium*	1.210 (–)
H_S_AE-4	*Aeromonas veronii*	KJ186903	*Aeromonas veronii*	2.077 (++)
H_S_AN-1	*Aeromonas veronii*	KJ186904	*Aeromonas veronii*	1.941 (+)
H_S_AN-2	*Aeromonas veronii*	KJ186905	*Aeromonas veronii*	2.070 (++)
K_G_AE-1	*Aeromonas veronii*	KJ186906	*Aeromonas hydrophila*	2.247 (++)
K_G_AE-2	*Aeromonas veronii*	KJ186907	*Aeromonas veronii*	2.077 (++)
K_G_AE-3	*Aquitalea denitrificans*	KJ186935	*Pseudomonas abietaniphila*	1.521 (–)
K_G_AE-4	*Enterobacter aerogenes*	KJ186945	*Citrobacter youngae*	1.848 (+)
K_G_AE-5	*Herbaspirillum lusitanum*	KJ186942	*Escherichia coli*	1.385 (–)
K_G_AN-1	*Aeromonas veronii*	KJ186908	*Aeromonas veronii*	2.068 (++)
K_G_AN-2	*Aeromonas veronii*	KJ186909	*Aeromonas veronii*	2.128 (++)
K_G_AN-3	*Aeromonas veronii*	KJ186910	*Aeromonas veronii*	2.062 (++)
K_G_AN-4	*Aeromonas veronii*	KJ186911	*Aeromonas jandaei*	2.180 (++)
K_G_AN-5	*Enterobacter aerogenes*	KJ186946	*Enterobacter hormaechei* ssp. *hormaechei*	1.506 (–)
K_S_AE-1	*Aeromonas veronii*	KJ186912	*Aeromonas veronii*	2.066 (++)
K_S_AE-2	*Aeromonas veronii*	KJ186913	*Aeromonas veronii*	2.124 (++)
K_S_AE-3	*Aeromonas veronii*	KJ186914	*Aeromonas sobria*	1.677 (–)
K_S_AE-4	*Aeromonas veronii*	KJ186915	*Aeromonas veronii*	2.000 (++)
K_S_AN-1	*Aeromonas veronii*	KJ186916	*Aeromonas veronii*	2.070 (++)
K_S_AN-2	*Aeromonas veronii*	KJ186917	*Aeromonas veronii*	2.017 (++)
K_S_AN-3	*Aeromonas veronii*	KJ186918	*Aeromonas veronii*	2.037 (++)
V_G_AE-1	*Herbaspirillum huttiense*	KJ186944	*Herbaspirillum huttiense*	1.618 (–)
V_G_AN-1	*Aquitalea magnusonii*	KJ186931	*Streptomyces phaeochromogenes*	1.486 (–)
V_G_AN-2	*Aquitalea magnusonii*	KJ186932	*Pseudomonas syringae* ssp. *syringae*	1.448 (–)
V_G_AN-3	*Aquitalea magnusonii*	KJ186933	*Gordonia terrae*	1.441 (–)
V_S_AE-1	*Aeromonas veronii*	KJ186919	*Aeromonas veronii*	2.128 (++)
V_S_AE-2	*Aeromonas veronii*	KJ186920	*Aeromonas veronii*	2.075 (++)
V_S_AN-1	*Aeromonas veronii*	KJ186921	*Aeromonas veronii*	2.060 (++)
V_S_AN-2	*Aeromonas veronii*	KJ186921	*Aeromonas veronii*	2.001 (++)
V_S_AN-3	*Aeromonas veronii*	KJ186923	*Aeromonas veronii*	2.222 (++)
V_S_AN-4	*Aeromonas veronii*	KJ186924	*Aeromonas veronii*	2.094 (++)

Explanations: C – Cep; H – Hejtman; K – Kanclíř; V – Veselí; G – gelled mass; S – superficial layer; AE – aerobic cultivation; AN – anaerobic cultivation. *Nearest phylogenetic neighbour determined by BLAST analysis; similarity higher than 99.5%. (+++) – highly probable species identification; (++) – secure genus and probable species identification; (+) – probable genus identification; (–) – no reliable identification.

mass was observed again in *P. magnifica* colonies sampled in Cep the pond.

Discussion

To the best of our knowledge, only marine bryozoans have previously been examined to determine their microbiota composition. This is therefore, the first study to focus on *P. magnifica* associated bacteria, and the numbers and species composition were determined. Because contamination with bacteria from surrounding water could not be completely prevented during the sampling, an important caveat is that not all cultured bacteria may be host specific or even symbi-

Table 3. The distribution (number of isolates and percentage) of bacterial species (as determined by *16S rRNA* sequence analysis) isolated from the superficial structure or gelled mass of *Pectinatella* colonies from four examined localities.

Bacterial species	Cep		Hejtman		Kanclíř		Veselí	
	Gelled mass	Superficies	Gelled mass	Superficies	Gelled mass	Superficies	Gelled mass	Superficies
Aeromonas veronii	1 (20.0%)	1 (12.5%)	1 (12.5%)	5 (83.3%)	6 (60.0%)	7 (100%)	0	6 (100%)
Aquitalea denitrificans	0	0	2 (25.0%)	0	1 (10.0%)	0	0	0
Aquitalea magnusonii	0	1 (12.5%)	4 (50.0%)	1 (16.7%)	0	0	3 (75.0%)	0
Enterobacter aerogenes	0	0	0	0	2 (20.0%)	0	0	0
Herbaspirillum huttiense	1 (20.0%)	0	0	0	0	0	1 (25.0%)	0
Herbaspirillum lusitanum	1 (20.0%)	0	0	0	1 (10.0%)	0	0	0
Chryseobacterium culicis	0	1 (12.5%)	0	0	0	0	0	0
Chryseobacterium gambrini	0	0	1 (12.5%)	0	0	0	0	0
Lactococcus lactis ssp. *lactis*	0	4 (50.0%)	0	0	0	0	0	0
Leuconostoc pseudomesenteroides	1 (20.0%)	0	0	0	0	0	0	0
Pseudomonas moraviensis	0	1 (12.5%)	0	0	0	0	0	0
Sphingomonas pituitosa	1 (20.0%)	0	0	0	0	0	0	0

otic. However, in this study, members of the genera *Aeromonas*, *Aquitalea*, *Chryseobacterium*, *Herbaspirillum*, *Enterobacter*, *Lactococcus*, *Leuconostoc*, *Pseudomonas* and *Sphingomonas* were found to be associated with *Pectinatella magnifica*. Listed genera were the most frequently isolated bacteria after cultivation when counts of culturable bacteria were determined in both the superficial and inner parts of the colonies. No trends in the bacterial numbers at each individual site during the season were observed. In most cases, higher bacterial numbers were found in surface layers reaching counts 4.95–6.61 and 2.30–6.52 log CFU/g for aerobes and anaerobes (including facultative anaerobes), respectively. More numerous microbiota in superficial layers may be expected because this part contains zooids with simple U-shaped gut that obtains nutrients by water filtration. During the summer season, zooids increase the colony volume by the production of a gelled mass. Despite the fact that the gelled mass of *Pectinatella* colonies was clear without visible turbidity, relatively high numbers of the bacteria were detected. The number of aerobic bacteria varied from 4.22 to 6.37 log CFU/g, with more variable counts determined after anaerobic cultivation (1.30–6.85 log CFU/g). There was no clear correlation between the bacterial number in *Pectinatella* colonies and the surrounding water. It was expected that the highest bacterial counts would be present in colonies sampled from water with the highest microbial contamination, but this was true only in some cases (Table 1). Moreover, the bacterial numbers in water varied during the season and no development in their counts were observed. This may be due to the fact that *Pectinatella* colonies filter the water, so the bacterial number may be higher in water where *Pectinatella* colonies are not yet fully developed or, alternately, in ponds where *Pectinatella* are too old and their colonies have already disintegrated.

Pectinatella magnifica associated bacteria were identified using two independent approaches. All isolates were successfully identified by the sequencing of the full *16S rRNA* gene and the similarity to nearest phylogenetic neighbour was higher than 99.5%. Less satisfactory results were achieved using MALDI-TOF analysis, in which only 1 strain was reliably identified to the species level and 30 strains achieved a secure genus and probable species identification. Twenty-nine strains were classified into the same species by both methods, 10 bacteria were identically identified only to the genus level using both approaches and in 15 isolates results differed. Because many strains were not reliably identified by MALDI-TOF, it can be recommended only as an auxiliary method, with the final taxonomic classification performed using a molecular approach.

The genera isolated from *Pectinatella* in this study had previously been described as ubiquitous in water, soil, plants and other environmental samples. *Aeromonas veronii*, a Gram-negative, facultative anaerobic rod-shaped Gammaproteobacterium, was the most abundant species in *Pectinatella*. These bacteria are ubiquitous in fresh water, and are found in association with a variety of vertebrates and invertebrates with both beneficial and pathogenic outcomes. They have been reported to cause wound infections and diarrhoea in humans and have been found as a pathogen and a gut symbiont in some fishes (Silver et al. 2011). *Ae. veronii* is most often associated with the digestive tract of the medicinal leech, where it maintains a symbiotic relationship with its host. It appears that *Ae. veronii* aids in the stabilization of microflora in the digestive tract of leeches because blood digested by this bacterium contains various antimicrobial substances (Indergand & Graf 2000). *Enterobacter aerogenes* also belongs to the Gammaproteobacteria. This Gram-negative, facultative anaerobic, rod-shaped bacterium was isolated from the gelled mass of *Pectinatella* sampled from Kanclíř pond. This species, like other Enterobacteriaceae, is generally found in the intestinal tract of animals but can be present as a contaminant in many natural environments including water, sewage, vegetables, and soil (Grimont & Grimont 2005). Another Gammaprotobacterium cultivated from the sampling site Cep was *Pseudomonas moraviensis*, a species originally isolated from the soil. Members of the genus are, again, ubiquitous in

soil, water and many other natural habitats (Tvrzová et al. 2006).

The second most frequently found bacterium in both the surface and gelled mass of *Pectinatella* colonies belonged to genus *Aquitalea*. The genus contains two species *Aq. magnusonii* (Lau et al. 2006) and *Aq. denitrificans* (Lee et al. 2009). Both of them were cultured from samples collected in all localities examined in our study. *Aquitalea* sp. are Gram-negative, rod-shaped, facultative anaerobic, non-spore-forming Betaproteobacteria. The first listed species was initially isolated from humic-lake samples collected from northern Wisconsin, USA (Lau et al. 2006), and the second was found in wetland peat collected from Yongneup, Korea (Lee et al. 2009). To our knowledge, this study is the first to find both species together. It appears that *Aquitalea* sp. may be a ubiquitous genus in waterlogged soils and fresh-water environment.

Herbaspirillum species (*H. lusitanum* and *H. huttiense*) were isolated from the inner gelled mass of colonies from three sampling sites (Cep, Kanclíř, Veselí). *Herbaspirillum* sp. are Gram-negative, aerobic, non-spore-forming, curved rods belonging to the Betaproteobacteria. The genus mainly comprises diazotrophic bacteria (*H. huttiense* does not fix nitrogen) with a potential endophytic and systemic colonization of many plants. Its presence has been demonstrated in the roots of several cereals and legumes, but bacteria belonging to this genus have been isolated from a variety of water samples as well (Valverde et al. 2003; Ding & Yokota 2004; Dobritsa et al. 2010).

The isolate producing yellow colonies on YTWG agar and showing highly viscous culture fluid after cultivation in a liquid version of the medium was identified as the Alphaproteobacterium *Sphingomonas pituitosa*. Morphological characteristics were also in accordance with the species description, as cells were Gram-negative, strictly aerobic, non-spore-forming, motile rods. *S. pituitosa* was first isolated from the eutrophic fountain in Vienna (Austria). Our strain was isolated from the gelled mass of a *Pectinatella* colony collected at the Cep pond. Members of the genus *Sphingomonas* are widely distributed in nature (water, soil, plant root systems etc.) and are able to survive in low nutrient concentrations. *Sphingomonas* spp. have been reported to have broad catabolic capabilities and for this reason have great potential for biotechnological applications in degradation, bioremediation and wastewater treatment. Bacteria of this genus are also able to produce sphingans, which are industrially useful exopolysaccharides (Denner et al. 2001).

One strain of *Chryseobacterium culicis* was isolated from the surface layer of a *Pectinatella* colony sampled from the Cep pond and one strain of *Chryseobacterium gambrini* was obtained after cultivation of the gelled mass from Hejtman pond. Members of this genus belong to the phylum Bacteroidetes and are Gram-negative, aerobic, non-spore-forming, non-motile rods. *Chryseobacterium* sp. have been reported to occur in aquatic environments, and many other niches, including activated sludge, soils and sediments, rhizospheres, as well as clinical and food processing samples. *Ch. culicis* was first isolated from the midgut of mosquitoes (Herzog et al. 2008; Kämpfer et al. 2010). Two species belonging to the Firmicutes group were found at the Cep site: *Leuconostoc pseudomesenteroides* and *Lactococcus lactis* ssp. *lactis*. Both of them are lactic acid bacteria typically inhabiting milk, dairy and other food but have commonly been detected in a variety of plant samples as well (Holzapfel et al. 2009; Teuber 2009).

Generally, there is lack of information about bacteria associated with the bryozoan species. Nevertheless, similar to marine bryozoans, the bacterial species composition of *P. magnifica* appears to be site rather than host specific. After comparing the composition of culturable bacteria isolated from different ponds in South Bohemia, we conclude that the examined *Pectinatella* are colonised by bacteria from the environment. *Aeromonas* and *Aquitalea* were found to be the most abundant genera in both the surface and gelled mass of *Pectinatella* at all locations and their symbiotic relationship with *P. magnifica* should not be excluded.

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5.3. Cultivable bacteria from *Pectinatella magnifica* and the surrounding water in South Bohemia indicate potential new Gammaproteobacterial, Betaproteobacterial and Firmicutes taxa

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RESEARCH LETTER – Taxonomy & Systematics

Cultivable bacteria from *Pectinatella magnifica* and the surrounding water in South Bohemia indicate potential new Gammaproteobacterial, Betaproteobacterial and Firmicutes taxa

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One sentence summary: Analysis of culivatable bacteria from *Pectinatella magnifica* and ambient water revealed 3 potential new genera and 15 potential new species.

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ABSTRACT

Pectinatella magnifica is a freshwater bryozoan, which has become a subject of scientific interest because of its invasive expansion worldwide. To obtain a comprehensive overview of its influence on environments, information on associated bacteria is needed. In this study, cultivable bacteria associated with *P. magnifica* were investigated. In total, 253 isolates were selected for preliminary identification by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and clustered based on repetitive extragenic palindromic-PCR profiles. Among these, 169 strains were selected and identified using 16S rRNA gene comparative analyses. The sequences were grouped into 76 phylotypes and affiliated with 67 species. The majority of isolated bacteria belonged to Gammaproteobacteria, followed by Betaproteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. Most strains within the Betaproteobacteria were isolated exclusively from bryozoan colonies. *Aeromonas* was the genus predominantly isolated from both *P. magnifica* and the water samples. Based on 16S rDNA similarity values, 15 putative new species belonging to the genera *Aeromonas*, *Aquitalea*, *Clostridium*, *Herbaspirillum*, *Chromobacterium*, *Chryseobacterium*, *Morganella*, *Paludibacterium*, *Pectobacterium*, *Rahnella*, *Rhodoferax* and *Serratia*, and putative new genera belonging to families *Clostridiaceae* and *Sporomusaceae* were revealed. The majority of the detected bacteria were species widely distributed in the environments; nevertheless, a possible symbiotic association of two new putative species with *P. magnifica* cannot be excluded.

Keywords: bacterial diversity; *Pectinatella magnifica*; Bryozoa; water sources; MALDI-TOF; REP-PCR

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INTRODUCTION

Pectinatella magnifica (Leidy, 1851) is a freshwater invertebrate resident within *Phylactolaemata*, a class of phylum *Bryozoa*. This so-called moss animal forms large gelatinous colonies on the surface of submerged substrates, or floats freely in the water bodies such as rivers, lakes, ponds and dams, and, as a filter feeder, it feeds mainly on plankton and detritus (Ingold *et al.* 1984; Balounová *et al.* 2011). This organism originates from North America (Lacourt 1968; Massard and Geimer 2008). Nevertheless, it was soon found in many other localities in Europe, Japan, and Korea, probably as a result of importation in freshwater tanks of ships from America (reviewed in Lacourt 1968). In water reservoirs of South Bohemia (Czech Republic), *P. magnifica* is an invasive species expanding massively since 2013. In recent years, an increasing number of studies have evaluated the invasive spread of this bryozoan from various countries (Balounová *et al.* 2013; Szekeres, Akác and Csányi 2013; Aleksandrov *et al.* 2014; Zorić *et al.* 2015) and its influence on aquatic environments and human health. This organism has been associated with biofouling (Wang, Wang and Cui 2017), viewed as an intermediate host for fish parasites, especially microsporidia (Canning *et al.* 2002), and considered a health risk resulting from the presence of cytotoxic agents (Kollar *et al.* 2016). In addition, *P. magnifica* was tested for its antibacterial and antifungal activity (Pejin *et al.* 2015). Bryozoan extracts were, in some cases, active against selected bacterial and fungal strains at concentrations lower than those of pure antibiotics, which served as controls. This finding contrasted with the results from a study by Kollar *et al.* (2016) that also demonstrated the antimicrobial activity of *P. magnifica* extracts, but at concentrations significantly higher than those in the previous study. Studies focused on the antimicrobial activity of freshwater bryozoans are rare, but marine forms are well known as producers of many biologically active secondary metabolites, which act as defensive agents against predators, parasites, and infections or as chemicals for intra- and inter-specific communication (Blackman and Walls 1995; Sharp, Winson and Porter 2007; Sinko *et al.* 2012). Some of these agents have interesting properties, which could be exploited pharmacologically (Pettit *et al.* 1982; Hennings *et al.* 1987; Milanowski *et al.* 2004; Prinsep *et al.* 2004). However, in many cases, the real sources of these compounds are not the bryozoans themselves, but their microscopic symbionts (Pettit *et al.* 1982; Schmidt *et al.* 2000; König *et al.* 2006), or possibly, epibiotic bacteria (Heindl *et al.* 2010). Although several studies have focused on *P. magnifica* from different perspectives, only one study described bacteria associated with this organism (Vlková *et al.* 2015). A search for cultivatable *P. magnifica*-associated bacteria is needed not only to discover producers of new antimicrobial compounds (Roca *et al.* 2015), but also to gain deeper insight into the ecology and interactions of the bacteria with their environment. There is no doubt that modern sequencing methods provide precise information about microbial diversity. This is especially true for microorganisms that are not capable of growing under experimental conditions and/or are only slightly distributed in the environment. On the other hand, isolation of bacteria in pure cultures under laboratory conditions is the first step to identify a new species. Cultivation enables study of the whole organism, including its phenotype, and provides the means to verify hypotheses arising from genomic data. Further, axenic cultures provide complete genomes of high quality that serve as references for a variety of meta-omics approaches and for the design of specific probes and primers. Even in the current 'omics' epoch, standard microbiological techniques remain a fundamental tool in microbiology, and studies referring the need of isolation and cultivation of aquatic microorganisms still arise (Giovannoni and Stingl 2007; Garcia 2016; Salcher and Šimek 2016). Hence, the aim of this study was to isolate and identify bacteria from *P. magnifica* colonies and the surrounding water collected in South Bohemia.

MATERIALS AND METHODS

Sample collection

Four water reservoirs in South Bohemia were chosen for sampling of *P. magnifica* colonies and the surrounding water: Hejtman (48°57′ N, 14°56′ E), Veselí (49°10′ N, 14°42′ E), Cep (48°56′ N, 14°52′ E) and Kanclíř (48°57′ N, 14°53′ E). From each locality, 12 colonies were obtained. A surface layer of bryozoan colonies containing zooids was collected separately from the inner jelly matter and aseptically transferred to vials containing oxygen-free peptone water (Oxoid, Basingstoke, UK) prepared by the Hungate technique (Hungate 1969). Water samples were collected in sterile vessels and held, along with colony samples, at 4°C until analysed. Samples analyses were performed within 5 h of collection.

Cultivation and enumeration of bacteria

Bacterial enumeration and isolation from *P. magnifica* colonies were performed using yeast extract-tryptone agar (YT, Oxoid, Basingstoke, UK) supplemented with 1 g/L of glucose dissolved in *Pectinatella* extract (YTGP) as described previously (Vlková *et al.* 2015). Briefly, for the extract preparation, whole colonies were boiled, filtered, and sterilised. Cold extract was used to support the growth of any symbiotic bacteria. Medium of the same composition dissolved in distilled water (YTGW) was used to enumerate bacteria from water samples.

Samples of *P. magnifica* colonies were homogenised in a blender, which simultaneously allowed bacterial cells to be dislodged from the colony structure, and serially diluted in oxygen-free peptone water. Cultivation was performed under both aerobic and anaerobic conditions. In order to differentiate anaerobic bacteria from facultative anaerobic bacteria, neomycin (70 mg/L) was added to the medium for obligate anaerobes to suppress the growth of facultative anaerobes (Pinheiro *et al.* 2003). Anaerobes and facultative anaerobes were cultivated in anaerobic jars (Anaerobic Plus System, Oxoid, Basingstoke, UK) in the presence of an AnaeroGen oxygen scavenging system (Thermo Fisher Scientific, Waltham, USA) at 24°C for 5 days, and aerobes were cultivated at the same temperature under aerobic conditions for 3 days. Bacteria from water samples were cultivated under the same conditions. After incubation, all colonies were counted and numbers expressed as log colony-forming units (CFU)/g.

From each cultivation variant, colonies differing in shape, margin, size, and pigmentation were picked and enriched in YTGP or YTGW broth. Isolates were then evaluated for their purity and selected based on morphological characteristics by using phase contrast microscopy (Nikon Instruments Europe B. V., Amsterdam, Netherlands). In total, 253 isolates were chosen for future identification.

Identification by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

Bacteria were identified based on ribosomal protein analysis on an Autoflex speed matrix-assisted laser desorption/ionisation

time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany), according to manufacturer's instructions. One millilitre of each fresh overnight culture was centrifuged for 3 min at 14 500× *g*, resuspended in 1.2 mL of 75% ethanol, and centrifuged again. The supernatant was discarded. This step was repeated twice to remove residues of the culture medium. The pellets were dried at room temperature and then resuspended in 10 μL of 70% formic acid and 10 μL of acetonitrile. After centrifuging for 2 min at 14 500× *g*, 1 μL of supernatant was transferred onto an MTP 384 polished steel BC target (Bruker Daltonik GmbH, Leipzig, Germany) and allowed to air dry. Each sample spot was overlaid with 1 μL of HCCA matrix solution (saturated solution of α-cyano-4-hydoxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid, Bruker Daltonik GmbH, Leipzig, Germany) and dried at room temperature. Spectra were measured automatically with FlexControl software version 3.4. To identify microorganisms, the raw data obtained from each isolate were imported into BioTyper software version 2.0 (Bruker Daltonik GmbH, Leipzig, Germany) and analysed. Measurements of each strain were performed in duplicate. Individual groups consisted of isolates, which were identified identically at species level, other groups were delineated according to the generic name and the last group consisted of isolates with a score values lower than 1.7. Within these groups, bacteria were clustered by repetitive extragenic palindromic (REP)-PCR.

DNA extraction and REP-PCR fingerprinting

Genomic DNAs were extracted using the PrepMan© Ultra Sample Preparation Reagent Protocol (Applied Biosystems Inc., Foster City, USA). For more detailed discrimination, bacterial isolates were characterised by REP-PCR. PCR amplification was performed using the primer $(GTG)_5$ [5′-GTGGTGGTGGTGGTG-3′] as described previously (Gevers, Huys and Swings 2001). The PCR protocol included initial denaturation at 95°C for 7 min, followed by 30 cycles of 90°C for 3 min, 40°C for 1 min, and 65°C for 8 min, and then a single final extension at 65°C for 16 min. PCRs were conducted in a volume of 25 μL containing 2 μL of DNA, 1 μL of primer (10 μM), 12.5 μL of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), and 9.5 μL of nuclease-free water (Thermo Fisher Scientific, Waltham, USA) in an automated T100 Thermal cycler (Bio-Rad, Hercules, USA). The PCR products were electrophoresed in a 1.5% agarose gel stained with GelRed (Biotium Inc., Fremont, USA) for 130 min at a constant voltage of 85 V in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, Fermentas Inc., Burlington, Canada), together with GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, USA) as a molecular marker. The REP-PCR fingerprints were visualised under a UV transilluminator (Bio-Rad, Hercules, USA), and digital images were captured. The resulting patterns were processed using BioNumerics software version 6.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). For the cluster analysis of these patterns, the Unweighted Pair Group Method with Arithmetic mean was applied with a distance tolerance 1.0% and optimisation set 1.0%.

Identification by 16S rRNA gene sequencing

A total of 169 bacterial strains were selected for identification using 16S rRNA gene comparative analyses. The genome region carrying the 16S rRNA gene (1490 bp) was amplified by PCR using forward primer fD1 [5′ AGA GTT TGA TCC TGG CTC AG 3′] and reverse primer rP2 [5′ ACG GCT ACC TTG TTA CGA CTT 3′] (Weisburg *et al.* 1991). The PCR conditions were as follows: initial denaturation at 92°C for 5 min, followed by 35 cycles of 92°C for 1 min, 52.5°C for 90 s, and 72°C for 2 min, followed by a single extension at 72°C for 5 min. PCRs were performed in a 25-μL volume containing 2 μL of DNA, 1 μL of each primer (10 μM), 12.5 μL DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), and 8.5 μL of nuclease-free water (Thermo Fisher Scientific, Waltham, USA) in an automated T100 Thermal cycler (Bio-Rad, Hercules, USA). The amplified PCR products were verified by agarose gel electrophoresis, purified with an illustra GFX PCR DNA Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK) and sequenced with each primer (fD1, rP2) using the Sanger method (GATC Biotech, Constance, Germany). Sequence data were aligned using the ClustalX package (Thompson *et al.* 1997) in BioEdit (Hall 1999) and compared with published sequences in the GenBank (at the National Center for Biotechnology Information, NCBI) nucleotide database using the BLAST program (Maidak *et al.* 2003) and EZ Taxon (Chun *et al.* 2007) to find the closest related taxa. The 16S rRNA gene sequences of representative strains were deposited in GenBank via the BankIt program at the NCBI website (https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank).

Phylogenetic analysis

Taxonomic relationships between isolates were evaluated by creating 16S rRNA-based phylogenetic trees. For tree reconstructions, isolates were divided into specific bacterial phyla. One representative isolate of each phylotype was selected for phylogenetic calculation. Obtained sequences and sequences of type strains of closely related taxa retrieved from the Ez Taxon database (Chun *et al.* 2007) were used for ClustalW alignment in Geneious 7.1.7 program (Biomatters Ltd., Auckland, New Zealand). Alignments were improved by elimination of poorly aligned positions and divergent regions using the Gblocks Server (Castresana 2000). The bootstrap method (1000 replications), Kimura 2-parameter model, and nearest-neighbour-interchange maximum likelihood algorithm were implemented in MEGA 6.06 software (Tamura *et al.* 2011) to construct the phylogenetic trees.

Statistical analysis

The bacterial counts were evaluated by one-way analysis of variance at a significance level of $P = 0.05$ using Statistica 12 (Stat-Soft Inc., Palo Alto, USA). Scheffé's pairwise comparison was used for post-hoc analysis. Differences in bacterial counts from both structures of the colonies and the surrounding water, as well as differences in counts between aerobic, anaerobic, and facultative anaerobic organisms were evaluated. Regression analysis was performed to reveal correlations among counts of bacteria present in bryozoan colonies and the surrounding water, $P = 0.01$.

RESULTS

Bacterial numbers

The average counts of cultivable bacteria from the surface layer and the jelly matter of *P. magnifica* colonies, and the surrounding water are listed in Table 1. The largest bacterial numbers of all three groups were found in the surface layer of *P. magnifica* colonies. In both, surface layer and jelly matter dominated aerobic bacteria, in surface layer together with facultative anaerobic bacteria. Regression analyses showed that the number of microorganisms in both parts of the *P. magnifica* colonies did not

Table 1. Counts of culturable bacteria in *Pectinatella magnifica* colonies and surrounding water (mean ± SD, n = 48), expressed as log CFU/g.

Type of samples	Aerobes	Facultative anaerobes	Anaerobes
Jelly matter	$5.13 \pm 0.76^{A,a}$	$4.63 \pm 0.95^{B,a}$	$3.78 \pm 1.35^{C,a}$
Surface layer	$6.90 \pm 1.22^{A,b}$	$7.38 \pm 1.20^{A,b}$	$4.51 \pm 1.43^{B,b}$
Water	$3.17 \pm 0.87^{A,c}$	$2.48 \pm 0.67^{B,c}$	$1.72 \pm 0.77^{C,c}$

^{A,B,C}values in lines with different superscript letters significantly differ ($P < 0.05$).
^{a,b,c}values in columns with different superscript letters significantly differ ($P < 0.05$).

correlate with the number of microorganisms in the ambient water.

Bacteria identification

We chose 253 pure strains with different cell and colony morphologies for identification. To gain preliminary information on affiliation to taxa, 198 isolates were analysed using MALDI-TOF mass spectrometry. The remaining isolates (n = 55) were difficult to culture after re-inoculation; therefore, they were analysed directly using a molecular-genetic approach. Identification was successful for 151 (76.3%) bacterial strains, of which 77 (38.9%) were identified to the species level and 74 (37.4%) to the genus level. Unreliable or misidentification results compared with those obtained from 16S rDNA and REP-PCR analyses were obtained in 47 cases (23.7%). More detailed results of the MALDI-TOF analysis are not shown because of the large amount of data. Bacteria were grouped according to the MALDI-TOF results. For the final identification by 16S rRNA gene sequencing, strains showing different REP-PCR fingerprint profiles were selected (n = 169). The REP-PCR fingerprinting method revealed the presence of identical strains, e.g. as in the cases of *Klebsiella oxytoca*, *Chryseobacterium* sp. and *Rhodoferax* sp. (Fig. S1, Supporting Information). Differences between strains in the same phylogenetic group were also observed. This is true especially for strains of *Aeromonas veronii* that showed high variability in their fingerprint profiles, which indicates considerable genotypic diversity within *A. veronii* population in the area (Fig. S2, Supporting Information). Table 2 shows representative strains (n = 169) of each phylotype revealed by 16S rRNA genes sequencing, NCBI accession numbers and similarities with phylogenetically nearest neighbours at a given length of base pairs, as well as the origin and number of isolates with identical 16S rRNA gene region. The closest related taxa and variability among isolates based on 16S rRNA gene similarities are shown in the phylogenetic trees (Figs 1–3). Because of the small number of Actinobacteria and Bacteroidetes representatives, phylogenetic trees of these phyla were not constructed, and their closest related taxa are described in followed text. Within Actinobacteria, PT132 was the most closely related to the type strain *Frigoribacterium faeni* 801 (Y18807) and PT8 to *Plantibacter flavus* VKM Ac-2504 (FXAP01000011). In the phylum Bacteroidetes, phylotype PT146 was nearest to *Chitinophaga niastensis* JS16-4 (EU714260), PT251 to *Chryseobacterium gambrini* 5-1St1a (AM232810) and PT238 to *C. indologenes* NBRC 14944 (BAVL01000024). Table 3 shows the abundance of detected bacterial species within the major phylogenetic groups and Fig. S3 (Supporting Information) displays distribution of bacterial genera. Based on species representation, *Aeromonas veronii* clearly predominated in both, *P. magnifica* and water samples.

Potential new taxa

For 15 phylotypes, the similarities of 16S rRNA gene sequence with those of validly described species were less than 98.65%, a threshold recommended for species delineation (Kim et al. 2014a). For higher taxa, pairwise identity values of almost full 16S rRNA sequences lower than 95% for genera, 92% for families, 89% for orders, 86% for classes, and 83% for phyla circumscription have recently been calculated. These thresholds are based on median values and the new taxa with higher similarity were validly described as well (Rosselló-Móra and Amann 2015). An example is a new genus described by Kim *et al.* (2014b) with similarity of 96.55%. Similarity 92.30% between PT59 and *Anaeroarcus burkinensis* (AJ010961) indicates that PT59 is a member of a new genus in the family *Sporomusaceae*. PT50 (94.12% with Y18189) and PT126 (96.15% with FR733677), similarly, are putative new genera in the family *Clostridiaceae* (Fig. 1). PT167 and PT168 were also affiliated with the family *Clostridiaceae*. Both were classified as *Clostridium* sp. based on similarities (97.42% and 97.79%) to their nearest neighbours PT167 (AY228334) and PT168 (AJ420008), respectively. In the Betaproteobacteria, seven new species in the genera *Aquitalea*, *Chromobacterium*, *Herbaspirillum*, *Paludibacterium* and *Rhodoferax* were identified. Two phylotypes, PT67 (97.71%) and PT99 (98.18%), were both closely related to *Aquitalea magnusonii* (DQ018117), but differed from each other at least in 16 nucleotides (ambiguous nucleotides due to the presence of diverse copies of 16S rDNA were not included) (Fig. 2). A more significant difference between phylotypes affiliated with the same species was observed for *Paludibacterium* spp. PT71 and PT78. Both were the most closely related to the type strain *Paludibacterium paludis* (HE981224), with similarities of 97.21% and 97.73%, respectively. However, PT71 and PT78 differed from one another in 34 nucleotides (97.43%), which indicate that they represent two separate taxa. *Chromobacterium* sp. PT79 (96.31% with AJ871127), *Herbaspirillum* sp. PT3 (98.47% with DQ188986), and *Rhodoferax* sp. PT111 (98.33% with AF084947) are other candidate representatives of new species. Within the phylum Gammaproteobacteria, a new *Pectobacterium* species might be represented by PT166 (98.56% with JN600322) and a new *Serratia* species by the PT83 (98.59% with AJ233428), see Fig. 3. Only one Bacteroidetes-related phylotype, PT238 *Chryseobacterium* sp., showed similarity lower that recommended threshold (98.47% with BAVL01000024).

The taxonomic tool based on 16S rRNA gene sequences does not have the resolving power to discriminate between very closely related taxa, as in the case of *Aeromonas* species. Interspecies 16S rRNA gene sequence similarity described in this genus can reach up of 100%, and other phylogenetic markers must be used to distinguish between bacterial species (Aravena-Román *et al.* 2013). The phylotype PT195 showed the closest similarity 98.84% with type strain *Aeromonas jandaei* (CDBV01000055), which is significant for the representatives of this group. Similar cases seem to be PT187 *Rahnella* sp. (98.77% with CP003244), and PT53 *Morganella* sp. (98.74% with DQ358146).

The majority of isolates that may represent members of new taxa originated from *P. magnifica* and belonged to the phyla Betaproteobacteria (n = 7), Firmicutes (n = 2) and Bacteroidetes (n = 1). Others were isolated from water samples and resided within Firmicutes (n = 5) or Gammaproteobacteria (n = 4). Phylotype PT50, belonging to Firmicutes, was found in both *P. magnifica* and water; therefore, it was counted in both groups of samples.

Table 2. 16S rDNA gene based identification.

		Accession no.	Similarity with nearest phylogenetic neighbour (%)[a]	Length (bp)	Source (no. of identical isolates)
Actinobacteria					
PT132	*Frigoribacterium faeni*	KY124232	99.35	1378	W (1)
PT8	*Plantibacter flavus*	KY124233	100	1303	W (1)
Bacteroidetes					
PT146	*Chitinophaga niastensis*	KY124229	98.93	1315	P (1)
PT251	*Chryseobacterium gambrini*	KY124230	99.93	1385	W (1)
PT238	*Chryseobacterium* sp.	KY124231	98.47	1375	P (3)
Firmicutes					
PT2	*Bacillus aerophilus*	KY124216	100	1430	W (1)
PT130	*Bacillus mycoides*	KY124218	100	1398	W (2)
PT13	*Bacillus subtilis*	KY124219	99.93	1414	P (1) W (1)
PT137	*Bacillus thuringiensis*	KY124217	100	1430	W (1)
PT161	*Carnobacterium maltaromaticum*	KY124220	99.93	1422	W (2)
PT50	*Clostridiaceae bacterium*	KY124215	94.12	1396	P (2) W (1)
PT126	*Clostridiaceae bacterium*	KY124225	96.15	1409	W (1)
PT110	*Clostridium frigidicarnis*	KY124222	99.78	1390	P (1)
PT62	*Clostridium ghonii*	KY124223	99.62	1330	P (1)
PT167	*Clostridium* sp.	KY124224	97.42	1362	W (1)
PT168	*Clostridium* sp.	KY124221	97.79	1309	W (1)
PT247	*Exiguobacterium acetylicum*	KY124226	99.93	1410	W (2)
PT88	*Lactococcus lactis* subsp. *cremoris*	KY124227	99.93	1416	W (1)
PT86	*Lactococcus lactis* subsp. *lactis*	KY124228	99.93	1412	W (2)
PT59	*Sporomusaceae bacterium*	KY124214	92.30	1315	P (1)
Betaproteobacteria					
PT133	*Aquitalea denitrificans*	KY124199	99.17	1319	W (1)
PT109	*Aquitalea magnusonii*	KY124202	99.32	1320	P (1)
PT67	*Aquitalea* sp.	KY124200	97.71	1396	P (1)
PT99	*Aquitalea* sp.	KY124201	98.18	1322	P (1)
PT61	*Chromobacterium haemolyticum*	KY124208	98.91	1286	P (1)
PT79	*Chromobacterium* sp.	KY124209	96.31	1412	P (2)
PT18	*Herbaspirillum aquaticum*	KY124203	99.93	1408	W (1)
PT149	*Herbaspirillum aquaticum*	KY124204	99.85	1363	P (1)
PT42	*Herbaspirillum hiltneri*	KY124205	99.32	1320	P (1)
PT217	*Herbaspirillum huttiense* subsp. *putei*	KY124206	100	1385	P (1)
PT3	*Herbaspirillum* sp.	KY124207	98.47	1370	P (5)
PT71	*Paludibacterium* sp.	KY124210	97.21	1327	P (2)
PT78	*Paludibacterium* sp.	KY124211	97.73	1323	P (1)
PT111	*Rhodoferax* sp.	KY124212	98.33	1399	P (3)
PT20	*Vogesella mureinivorans*	KY124213	99.47	1321	W (1)
Gammaproteobacteria					
PT212	*Acinetobacter pittii*	KY124158	100	1354	P (2)
PT14	*Aeromonas allosaccharophila*	KY124159	100	1329	W (1)
PT154	*Aeromonas allosaccharophila*	KY124160	100	1407	P (1)
PT91	*Aeromonas australiensis*	KY124161	99.86	1411	P (9)
PT158	*Aeromonas eucrenophila*	KY124162	99.86	1384	W (1)
PT162	*Aeromonas eucrenophila*	KY124163	100	1410	W (1)
PT170	*Aeromonas finlandiensis*	KY124172	99.86	1415	P (5)
PT52	*Aeromonas hydrophila* subsp. *ranae*	KY124164	99.93	1378	W (1)
PT29	*Aeromonas jandaei*	KY124165	99.85	1376	P (2)
PT178	*Aeromonas media*	KY124167	99.85	1370	P (1)
PT169	*Aeromonas popoffii*	KY124168	99.93	1409	P (1) W (1)
PT195	*Aeromonas* sp.	KY124166	98.84	1376	W (2)
PT22	*Aeromonas veronii*	KY124169	100	1404	P (20) W (6)
PT55	*Aeromonas veronii*	KY124170	99. 64	1412	P (3)
PT97	*Aeromonas veronii*	KY124171	99.86	1388	P (20) W (5)
PT160	*Citrobacter gillenii*	KY124173	99.55	1325	W (2)
PT47	*Cosenzaea myxofaciens*	KY124174	100	1417	W (2)
PT51	*Dickeya zeae*	KY124175	99.25	1332	W (1)
PT12	*Enterobacter asburiae*	KY124176	99.93	1373	P (1) W (1)
PT129	*Erwinia persicina*	KY124177	100	1325	W (1)

Table 2 *continued*

		Accession no.	Similarity with nearest phylogenetic neighbour (%)[a]	Length (bp)	Source (no. of identical isolates)
PT157	*Flavobacterium acidificum*	KY124178	99.62	1325	W (1)
PT72	*Haemophilus piscium*	KY124179	100	1413	P (1) W (1)
PT58	*Klebsiella michiganensis*	KY124180	100	1324	P (1)
PT164	*Klebsiella oxytoca*	KY124181	100	1324	W (3)
PT81	*Kluyvera ascorbata*	KY124182	99.55	1325	W (1)
PT218	*Lelliottia amnigena*	KY124183	99.70	1327	P (2)
PT156	*Morganella psychrotolerans*	KY124185	99.70	1394	W (1)
PT53	*Morganella* sp.	KY124184	98.74	1412	W (1)
PT15	*Pantoea agglomerans*	KY124186	100	1323	W (1)
PT166	*Pectobacterium* sp.	KY124187	98.56	1,33	W (1)
PT19	*Plesiomonas shigelloides*	KY124188	100	1418	W (1)
PT87	*Plesiomonas shigelloides*	KY124189	99.93	1388	W (1)
PT127	*Plesiomonas shigelloides*	KY124190	99.93	1415	W (2)
PT233	*Plesiomonas shigelloides*	KY124191	100	1373	P (2)
PT245	*Pseudomonas baetica*	KY124192	99.71	1387	W (1)
PT70	*Pseudomonas geniculata*	KY124193	100	1382	P (1)
PT148	*Pseudomonas protegens*	KY124194	100	1390	P (1)
PT187	*Rahnella* sp.	KY124195	98.77	1384	W (1)
PT135	*Rahnella* sp.	KY124196	99.85	1388	W (1)
PT83	*Serratia* sp.	KY124197	98.59	1416	W (1)
PT39	*Shewanella xiamenensis*	KY124198	99.35	1380	P (1)

[a] Compared with type strain obtained from Ez Taxon.
P—*Pectinatella magnifica*, W—water, bp—base pairs.

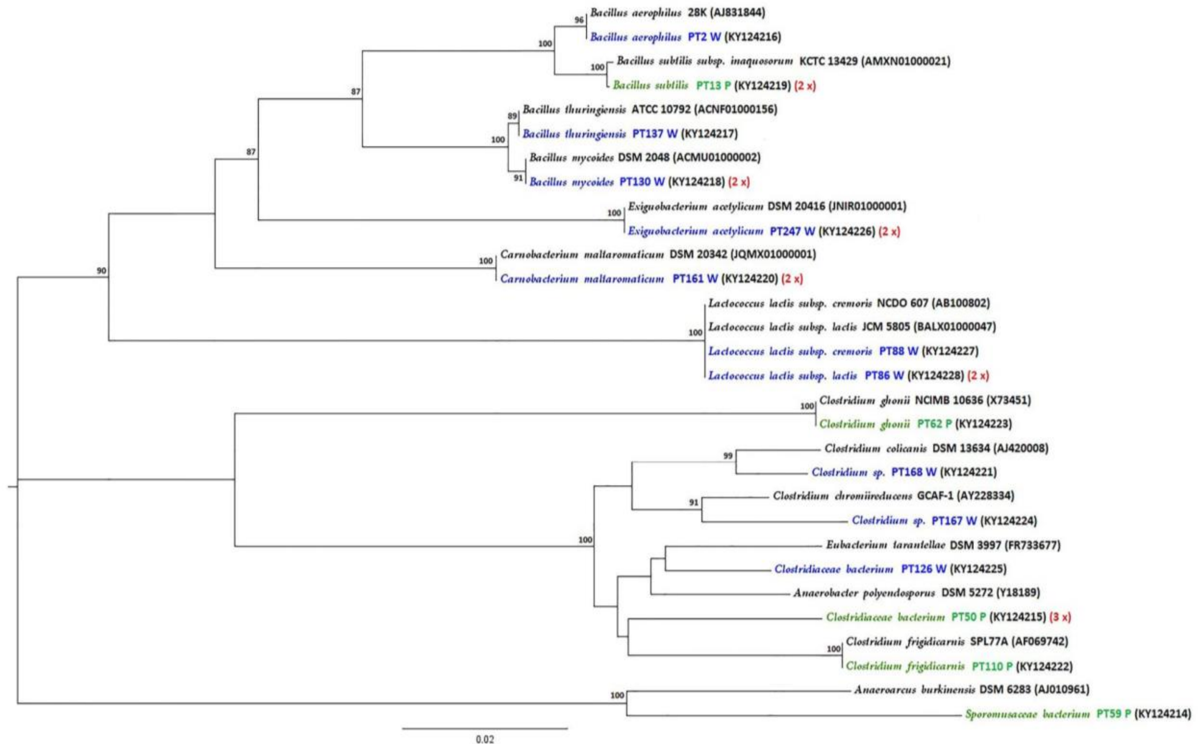

Figure 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1192 nt) revealing relationships among Firmicutes isolates originated from *Pectinatella magnifica* biomass (green) and surrounding water (blue). Numbers of isolates with identical sequences are marked (red). Type strains of most closely taxa together with the GenBank accession numbers are also included (black) for comparison. Bootstrap values, expressed as percentages of 1000 data sets ($\geq$70), are given at nodes. Numbers in parentheses of bacterial isolates correspond to the GenBank accession numbers. The tree was reconstructed as unrooted. Bar, 0.02 substitutions per nucleotide position.

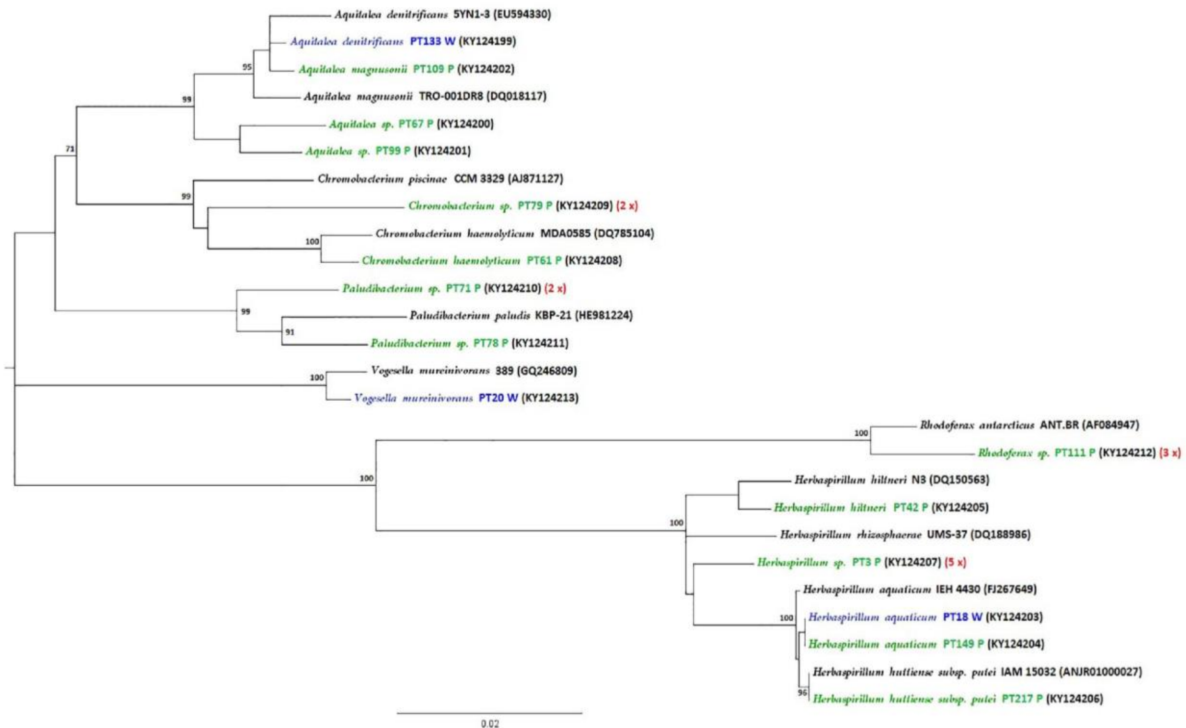

Figure 2. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1265 nt) revealing relationships among Betaproteobacteria isolates originated from *Pectinatella magnifica* biomass (green) and surrounding water (blue). Numbers of isolates with identical sequences are marked (red). Type strains of most closely taxa together with the GenBank accession numbers are also included (black) for comparison. Bootstrap values, expressed as percentages of 1000 data sets (≥70), are given at nodes. Numbers in parentheses of bacterial isolates correspond to the GenBank accession numbers. The tree was reconstructed as unrooted. Bar, 0.02 substitutions per nucleotide position.

DISCUSSION

To the best of our knowledge, there are no studies focused on bacteria inhabiting freshwater bryozoans or evidence of their possible specific interactions with the animal. Only one publication describes *P. magnifica*-associated bacteria (Vlková *et al.* 2015). Similar to our results, *Aeromonas* strains were the most commonly isolated bacteria, followed by *Aquitalea*. Microbial diversity of cultivatable bacteria associated with the marine bryozoan *Flustra foliacea* were examined (Pukall *et al.* 2001). Nevertheless, a comparison of our results with those of studies focused on microorganisms associated with marine forms is not fully relevant, because bacteria that grow in oceans and freshwater are frequently evolutionary distinct (Zwart *et al.* 2002). On the other hand, it was concluded that the *F. foliacea* accepts colonisation of surface by bacteria that are inhabitants of marine environments and have been transferred to this environment from terrestrial sites (Pukall *et al.* 2001). This supports with our results. Most bacterial species isolated from *P. magnifica* were also found in water and/or are described as bacteria commonly found in the environment.

The highest bacterial counts were expected to be found in colonies sampled from water with the highest microbial content, but this was true only in some cases. Statistical analysis showed that the numbers of microorganisms in samples of *P. magnifica* did not correlate with those in the surrounding water. This finding could be indicative of the commensal or symbiotic character of these organisms. Specific bacterial communities would be strongly dependent on its host's attributes, which are difficult if not impossible to simulate *in vitro*. In this study, the growth of specific bacteria was supported by adding of *P. magnifica* extract to the culture medium. Most bacteria belonging to the beta subclass of Proteobacteria were isolated exclusively from *P. magnifica* colonies, and 7 out of 12 species detected were classified as potential new taxa. For this reason, we can assume that bacteria living in symbiosis with *P. magnifica* may belong to this phylum. This presumption is supported by the fact that five strains of putative novel species *Herbaspirillum* sp. PT3 were found in the bryozoan colonies at three different locations, but none was detected in water samples. On the other hand, the 16S rRNA gene sequence of *Oxalobacteraceae* bacterium PRE7E (KM187616), associated with the skin of the amphibian *Notophthalmus viridescens*, was identical to our strain PT3. *Aquitalea* spp. PT67 and PT99 were similar cases. Both phylotypes were very closely related to undescribed *Neisseriaceae* bacteria associated with the skin of the amphibian *Rana catesbeiana* (phylotype PT67 to isolate BFE64E [KM187092] and PT99 to BFE40G [KM187049]). Both amphibians, like *P. magnifica*, are native to North America, and all three mentioned sequences of amphibian-associated bacteria were obtained from samples in North America in the same study (Walke *et al.* 2015). Therefore, the question of whether these species could be distributed together with bryozoans may arise. Questionable is specific association of PT78 because of its relatedness to *Paludibacterium* sp. J8SN6 (JF327660), which was detected in soil. Slightly distant from the closest *Paludibacterium* sp. CRh31 (KR780468), isolated from Dongxiang wild rice, was strain PT71. Although the sequences were not identical and specific adaptation cannot be fully excluded, *Paludibacterium* spp. (still undescribed) seems to be common environmental bacteria rather than host-specific symbionts. This is valid also of *Rhodoferax* sp. PT111 and

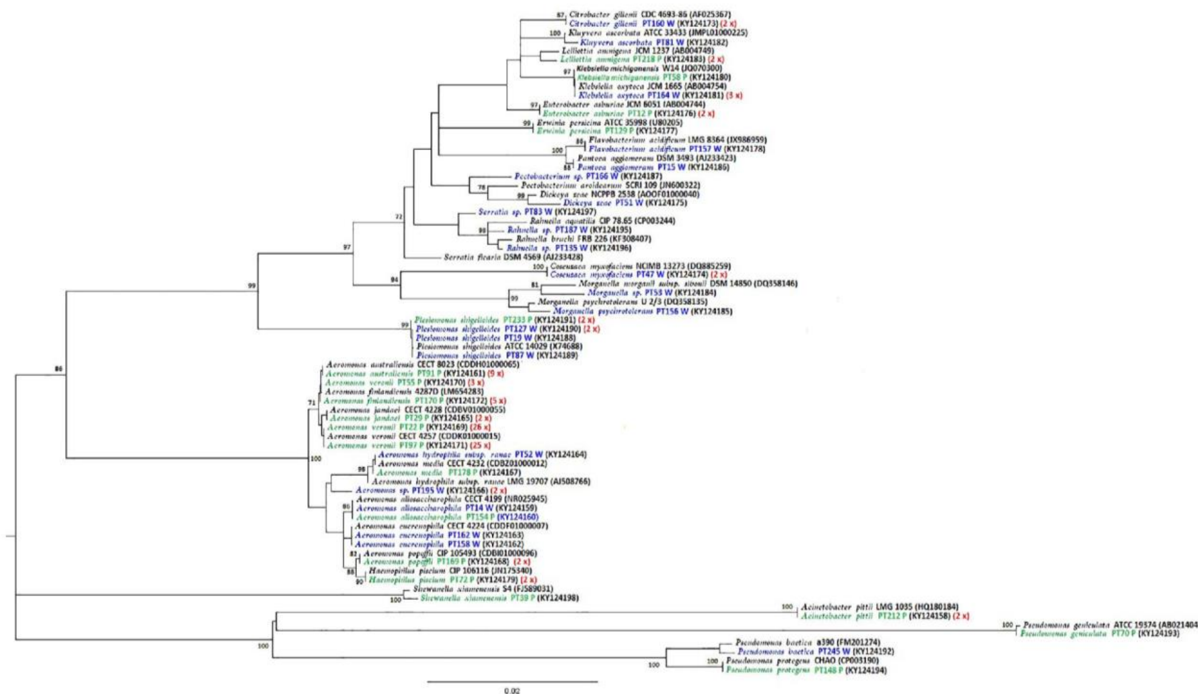

Figure 3. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1262 nt) revealing relationships among Gammaproteobacteria isolates originated from *Pectinatella magnifica* biomass (green) and surrounding water (blue). Numbers of isolates with identical sequences are marked (red). Type strains of most closely taxa together with the GenBank accession numbers are also included (black) for comparison. Bootstrap values, expressed as percentages of 1000 data sets ($\geq$70), are given at nodes. Numbers in parentheses of bacterial isolates correspond to the GenBank accession numbers. The tree was reconstructed as unrooted. Bar, 0.02 substitutions per nucleotide position.

Chryseobacterium sp. PT238. Bacteria of genus *Rhodoferax* have been found in high numbers in a wide range of freshwater habitats (Salcher and Šimek 2016). Among our strains, the most probable *P. magnifica* symbiont seemed to be Betaproteobacterial species PT79, classified as *Chromobacterium* sp. Its 16S rRNA gene sequence similarity with all published cultured and uncultured bacteria was 97% or less. Further, microbial symbionts of marine bryozoan have been found to be involved in chemical defence of their hosts by production of bioactive metabolites. The antimicrobial activity, and/or cytotoxicity are regarded as the most significant properties of these substances (Sharp, Winson and Porter 2007) and the ability to produce drugs with these effects is a characteristic trait of many *Chromobacterium* spp. (Durán and Menck 2001; Han, Han and Segal 2008). In addition, *Sporomusaceae* bacterium PT59 was also found only in *P. magnifica* colonies.

We expected greater species diversity in *P. magnifica* than in water samples because of the bryozoan filter feeding strategy, but this was not confirmed in this study. Certainly, this finding is not conclusive. Leaving aside that this study was restricted by the cultivation technique with all of its drawbacks (Salmonová and Bunešová 2017), a simple explanation may be indicated by the frequency of the ubiquitous bacterium *Aeromonas veronii*. *Aeromonas veronii* strains were the most frequently isolated bacteria in our water samples. Although it was not clearly predominant in water, filtration probably allowed the accumulation of cells and increased the prevalence of *A. veronii* in bryozoan colonies. Increases in the total counts of cultivable bacteria make isolation of less numerous groups from lower dilutions difficult or impossible. Moreover, as facultative anaerobes (Seshadri *et al.* 2006), aeromonads are able to grow under both aerobic and anaerobic conditions, without being suppressed by neomycin at the concentration of 70 mg/L. *Aeromonas veronii* group have been reported to inhabit a wide range of vertebrate and invertebrate host animals as a symbiont or pathogen (Silver *et al.* 2011). Therefore, significant predominance of these bacteria in *Pectinatella* colonies might be caused not only by accumulation, but also due to specific interactions. From the second perspective, *Aeromonas* spp. have been implicated in the wide spectrum of human and fish diseases (Beaz-Hidalgo and Figueras 2013). Massive expansion of *P. magnifica* could pose a health risk and economic loss in recreational and breeding areas. It is worth noting that the predominance of *Aeromonas* species (determined by MALDI-TOF) was observed prior to the selection of bacteria for sequencing. Despite this fact, we revealed 18 potential new taxa, 10 of which were isolated from bryozoan colonies and 9 of these exclusively.

Table 3. Abundance of detected bacterial species within major phylogenetic groups (determined by combination of MALDI-TOF, REP-PCR and 16S rDNA gene sequencing results).

	Number (%) of isolates	
Phylogenetic group	*Pectinatella* samples	Water samples
Actinobacteria	0	2 (3.08)
Bacteroidetes	4 (3.85)	1 (1.54)
Firmicutes	6 (5.77)	16 (24.62)
Betaproteobacteria	20 (19.23)	3 (4.62)
Gammaproteobacteria	74 (71.15)	43 (66.15)

CONCLUSIONS

Our study showed that *P. magnifica* is a prolific source of hitherto unknown bacteria that are able to grow easily under laboratory conditions. *Pectinatella magnifica* is colonised predominantly by allochthonous species, either described as bacteria widely spread in the environment or related to less common bacteria, but detected in environmental samples other than in bryozoans. We can say that *P. magnifica* acts as a biofilter that captures bacteria present in water, and accumulates them to relatively high counts. This may be useful for the isolation of a new species, which are not detected in water because of their low concentrations, although we found potential new taxa even in water. We revealed a total of 18 bacterial phylotypes, representing 15 putative new species and 3 putative new genera. Two strains were detected exclusively in our samples of *P. magnifica* colonies, and a symbiotic association between these bacteria and the bryozoan could not be excluded.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflict of interest. None declared.

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5.4. Invasion of *Pectinatella magnifica* in freshwater resources of the Czech Republic

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Invasion of *Pectinatella magnifica* in Freshwater Resources of the Czech Republic

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Abstract**—***Pectinatella magnifica* (Leidy, 1851) is an invasive freshwater animal that lives in colonies. A colony of *Pectinatella magnifica* (a gelatinous blob) can be up to several feet in diameter large and under favorable conditions it exhibits an extreme growth rate. Recently European countries around rivers of Elbe, Oder, Danube, Rhine and Vltava have confirmed invasion of *Pectinatella magnifica*, including freshwater reservoirs in South Bohemia (Czech Republic). Our project (Czech Science Foundation, GAČR P503/12/0337) is focused onto biology and chemistry of *Pectinatella magnifica*. We monitor the organism occurrence in selected South Bohemia ponds and sandpits during the last years, collecting information about physical properties of surrounding water, and sampling the colonies for various analyses (classification, maps of secondary metabolites, toxicity tests). Because the gelatinous matrix is during the colony lifetime also a host for algae, bacteria and cyanobacteria (co-habitants), in this contribution, we also applied a high performance liquid chromatography (HPLC) method for determination of potentially present cyanobacterial toxins (microcystin-LR, microcystin-RR, nodularin). Results from the last 3-year monitoring show that these toxins are under limit of detection (LOD), so that they do not represent a danger yet. The final goal of our study is to assess toxicity risks related to fresh water resources invaded by *Pectinatella magnifica*, and to understand the process of invasion, which can enable to control it.

Keywords**—**Cyanobacteria, freshwater resources, *Pectinatella magnifica* invasion, toxicity monitoring

I. Introduction

PECTINATELLA MAGNIFICA (Leidy, 1851) (*PM*) is a bryozoan species spreading with an invasive character. A native area of the animal is the east part of the Mississippi River, from Ontario to Florida, where it was observed already in 19th century. Since then, *PM* has been spreading also to Korea, India, Japan, and Turkey. In Europe, it has been recorded in Germany, Romania, Turkey, and France. Its occurrence in the Netherlands has been first reported in 2003. The newest discoveries are for the Rhine basin in the area between Luxembourg and Germany. Recently, it was also recorded in the Czech Republic [1].

A colony of *Pectinatella magnifica* is built from a gelatinous matrix covered by hundreds of individual filter feeding zooids. Owing to its massive occurrence, it presumably has an important influence on the ecosystem (species composition, trophic level, and hydro-chemistry). Large *PM* colonies can also clog water intake and irrigation pipes creating economic and engineering challenges. Therefore, the implication of *PM* invasion in the freshwater reservoir concerns not only biology but also ecology, planning of further usage of water supply resources and thus the quality of life.

The knowledge about chemistry of *PM*, about its second metabolites, and also the recognition of the state, development and plankton dynamics (which affects the food chain) of sandpits, ponds and rivers with *PM* colonies is a key for understanding and controlling the invasion.

In order to understand the invasion in a complex way, this paper consists of three separated parts concerning i/ statistical evaluation of *PM* spread during the season 2015, ii/ analysis of bacteria related to *PM* and microbiological activity of *PM* extracts, iii/ selected chemical analysis of lyophilized *PM* samples.

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II. Statistical Evaluation of *Pectinatella magnifica* Colonies in Area Třeboňsko, 201 Seasons

A. Invasion of Pectinatella magnifica in Area Třeboňsko: Biomass Production

In the Protected Landscape Area and Biosphere reserve "Třeboňsko" the bryozoan was first found in 2003 in a mesotrophic sandpit. Then, the species gradually spread to many other sites on the Třeboňsko area and at most of these locations its occurrence has invasive properties [2].

The most important parameter affecting *PM* occurrence is the low trophicity of water, thus it expanded to some gravel sandpits and fishponds without intensive pisciculture. Colonies of this invasive bryozoan in this area are found typically on submerged branches of willow trees [3].

Biomass, expressed by weight and the number of colonies, is an important factor in terms of the potential production of biologically active substances. Higher biomass production means a higher amount of zooids, thus more metabolically active units of *Pectinatella magnifica*. In addition, a larger amount of biomass means higher volume of the matrix that is colonized by other potential producers of biologically active substances: algae, cyanobacteria and bacteria. Therefore, the monitoring of amount of biomass is one of the basic work for following studies.

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B. Statistical Methods

1) Data Collection Methods

Sample collection was carried out at five localities – two gravel sandpits of "Cep" and "Veselí I", and three ponds of "Podřezaný", "Hejtman", and "Nový Kanclíř". Six transects were chosen on each locality in the length of 10 m along the shore and of width 5 m. All colonies found in these transects were measured and weighted. Sample collection was duplicated on identical transects in July 2015 (first sampling) and August 2015 (second sampling).

2) Data Analysis Methods

The exploratory data analysis was performed by bar charts and the interference analysis by analysis of variance. A Kruskal-Wallis test was chosen, as the data were not normally distributed.

The localities were used as the independent variables, while the dependent ones were the biomass, number of colonies and the average colony mass. The null hypotheses were designed so, that the biomass/number of colonies/average colony mass do not differ between the colonies. The critical significance level was set equally for all analyses comparing the dependent variables to α=0.05. For comparing the dependent variables this significance level was adjusted following Bonferroni to α=0.02. Next, the H-statistics were computed and compared to the critical value $\chi2$ — distribution with m-1 degrees of freedom. The null hypothesis was rejected, whenever the statistical value was higher than the value of $\chi2$ — distribution with m-1 degrees of freedom.

C. Results

1) The Exploratory Data Analysis

Biomass

A total amount of biomass was higher for the first sample collection in July 2015 at all of the localities. On Hejtman, Cep and Veselí I, no colonies were found during the second collection and on Nový Kanclíř only one colony was found in August, weighing 1.7 kg. The reason for the lack of colonies in the second term of collection of 2015 were probably unusual climatic conditions (see Conclusions, part D).

The biggest biomass from the collection in July (129.4 kg) was found on pond Nový Kanclíř. Quite high level of biomass (compared to previous years) was found on the pond Podřezaný (409 kg). It was also considerably higher than the biomass found on other localities – Veselí I (41 kg), Hejtman (25 kg) and Cep (0.6 kg).

Colony Number

The highest number of colonies within the first collection was found on the gravel sandpit Veselí I (282), and on Nový Kanclíř (257), significantly lower on Hejtman (48) and the lowest on Cep (12). Within the second collecting in August, no colonies were found on three localities (Hejtman, Cep and Veselí I). On the pond Nový Kanclíř, only a single colony was found. Considerably higher amount within the second sampling was only found on Podřezaný pond (450 colonies in comparison to 258 in the first sampling).

Average Colony Mass

The average colony mass was calculated as a ratio of the cumulative mass collected in the six transects at a given locality divided by the total number of colonies counted there. The highest average colony mass from the first sampling was recorded on the pond Podřezaný (1.58 kg), huge colonies with the average weight of 1.08 kg were found also on Nový Kanclíř. The average colony mass on Hejtman was 0.52 kg, and the lowest on the gravel sandpit Cep (0.05 kg).

Average colony masses for the five localities in the season 2015 are plotted in Fig. 1 (white bars). Due to the lack of colonies in August 2015 on most of the localities, the second collection was not considered for the calculation of an average. For comparison, there adequate data from previous season (2014) were added to the graph (grey bars).

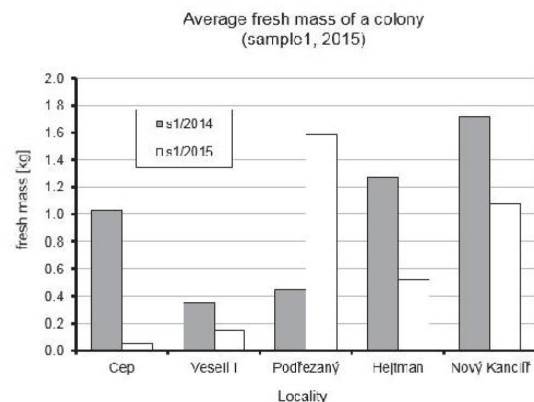

Fig. 1 Comparison of the average fresh mass of a *PM* colony at five localities in season 2015 (white bars) to the previous season 2014 (grey bars). The first two localities are gravel sandpits; the others are ponds. Due to extraordinary weather in summer 2015 (see text), instead of average values from two sampling dates, only the first sampling was considered.

2) Interference Analysis

Biomass

The null hypothesis was that the amount of biomass is the same on all localities. This hypothesis was rejected on a significance level α=0.05 (H=20.64, $\chi2$=11.07). The amount of biomass differs greatly within the localities and specific locality has a big influence on the biomass production. After a simultaneous comparison of the localities on a significance level α=0.02, the same significant difference in the biomass production on all of the localities was found.

Number of Colonies

Data of this factor were the same as of the previous one. The null hypothesis that the number of the colonies is the same on all localities was rejected with the level of significance α = 0.05 (H=55.92, $\chi2$=11.07) and with a simultaneous comparison of the localities on a significance level α = 0.02 a significant difference in the number of

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colonies was found.

Average Colony Mass

The situation with this parameter is also the same as for the other parameters. That means a considerable dissimilarity between the average colony mass on the studied localities. The null hypothesis was rejected on the level of significance $\alpha = 0.05$ ($H=113.79$, $\chi 2=11.07$) as well as simultaneous comparison of the localities on a significance level $\alpha = 0.02$ found a significant difference in the average colony mass within all of the localities.

D. Conclusions

The season of 2015 varied from the previous ones primarily because of lack of colonies at the second sampling (August 2015) at three of five localities and because of a great increase of biomass on the pond Podřezaný in comparison with previous years. An explanation can be the extraordinary weather course: a period of high temperatures and drought caused an increase of trophicity in most freshwaters. The exception was a pond Podřezaný, into which less eutrophic water flows from the forest.

Preliminary statistical results showed dissimilarity of biomass production on different localities and also dissimilarity of biomass production between seasons 2015 and 2014. Study that is more detailed will be needed to correlate factors of *PM* invasive spreading within a longer time period.

III. Microbiota Associated with *Pectinatella magnifica* and Its Antimicrobial Activity

Because marine bryozoan are harbored by symbiotic bacteria which are known for the production of antimicrobial [4]–[5] and other bioactive compounds [6]–[7], there is a presumption that also freshwater *P. magnifica* may serve as a host for some specific bacterial species which may possess antimicrobial activity. Hence, the aim of our part of the study was to enumerate and isolate culturable bacteria from *PM* colonies and test their antimicrobial activity.

TABLE I
BACTERIAL NUMBERS DETERMINED IN *PECTINATELLA MAGNIFICA* COLONIES AND WATER

Type of bacteria	Type of sample	No. of samples	Bacterial counts (log CFU/g or mL)	
			Varied from to	Median
Aerobic	Gelled mass	144	3.64 – 6.65	5.01
	Superficial layer	144	4.96 – 9.51	6.64
	Water	48	1.88 – 5.12	3.23
Facultative anaerobic	Gelled mass	144	3.00 – 6.26	4.53
	Superficial layer	144	4.91 – 9.20	7.51
	Water	48	1.20 – 3.63	2.42
Anaerobic	Gelled mass	144	1.00 – 6.85	3.65
	Superficial layer	144	1.74 – 7.90	4.15
	Water	48	1.00 – 2.96	1.75

A. Material and Methods

Samples of *PM* colonies and surrounding water were collected at four locations: two ponds "Hejtman" and "Kanclíř", two gravel sandpits "Cep" and "Veselí I". Three colonies from each location were collected at three different sampling times during the years 2012–2015. The superficial layer of bryozoan colonies containing zooids was collected separately from the inner gelled mass. Samples were aseptically transferred to vials containing oxygen-free peptone water, kept in a refrigerator and analyzed within 5 hours after collection. Water samples were collected into a sterile vessel and kept cold at 4 °C until analyzed.

Bacterial counts were determined by cultivation on yeast extract-tryptone agar (YT; Oxoid) supplemented with 1 g/L of glucose (yeast-tryptone-glucose agar; YTG). Samples were homogenized and serially diluted in peptone water under anaerobic conditions. One mL of appropriate dilutions was transferred to sterile Petri dishes, which were immediately filled with YTG agar. Cultivation was carried out at both, aerobic and anaerobic (Anaerobic Plus System; Oxoid) conditions at 25 °C for 3 days. Under the anaerobic condition, also facultative anaerobes were able to growth. So, for the determination of strictly anaerobic bacteria, neomycin was added to the media at a concentration 70 mg/L to avoid the growth of facultative anaerobes. After incubation, bacterial colonies were counted and data were expressed as a log CFU/g. Bacterial colonies representing a wide range of pigments and colony types were picked up from each cultivation variant, enriched in YTG medium and identified [8]. Bacteria in water samples were enumerated in triplicate using YTG agar, cultivation was performed under the same conditions as described above.

Antimicrobial activity of cell-free supernatants from *PM* associated bacteria against potential pathogens, meat-spoiling psychrophilic bacteria, intestinal bacteria and bacteria isolated from *PM* was tested by agar-well diffusion method. Cell-free culture supernatants were obtained from *Acinetobacter pitii* (2 stains), *Aeromonas veronii* (12 strains), *Aquitalea denitrificans* (10 strains), *Aquitalea magnusonii* (5 strains), *Enterobacter* sp. (3 strains), *Klebsiella* sp. (2 strains) *Lactococcus lactis* ssp. *lactis* (2 strains), *Leuconostoc pseudomesenteroides* (1 strain), *Pseudomonas* sp. (5 strains), and *Sphingomonas pituitosa* (2 strains) by microcentrifugation (18 000 x g, 4 °C, 20 min) of overnight bacterial cultures. 20 mL of nutrient agar was added to 1 mL of overnight cultures of potential pathogenic, meat-spoiling, intestinal and *PM* associated bacteria (tested strains: 5 strains of *E. coli*, 3 strains of *Salmonella* sp., 7 strains of *Clostridium* sp., 3 strains of *Lactobacillus* sp., 2 strains of *Bifidobacterium* sp., *Acinetobacter parvus* CCM7030, *Moraxella canis* CCM4590, *Pseudomonas aeruginosa* CCM1960, *Pseudomonas fluorescence* CCM2115, *Propionibacterium acnes* DSMZ183, *Serratia marcescens* DSMZ30212, *Micrococcus luteus* ATCC10240, *Listeria monocytogenes* ATCC7644, and 15 strains isolated from *PM* colonies). Plates were dispersed and agar allowed to firm. Antimicrobial tests were done under both, aerobic and anaerobic conditions and different cultivation media were used. The first one was the basic medium appropriate for each tested bacterium; the second medium was prepared from *PM* water extract, and the last one contained blood. Wells in the agars were created using a 6-mm

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using the column chromatography and preparative TLC on silica. Column chromatography was carried out on Merck silica gel 60 (particle size 0.040-0.063 mm). TLC plates of Silicagel 60 F_{254} (Merck) (UV detection at 254 and 366 nm, and spraying will sulphuric acid and heating to 100 °C, respectively) were employed. Different combinations of solvents were used to formulate the mobile phases suitable for successful isolation of content compounds. Tested were many combinations of chloroform, ethyl acetate, hexane, acetone, benzene, and ethyl acetate with addition of formic acid in ratios of 50:50, 60:40, and 80:20 (v/v). Fractions obtained by elution with selected mobile phases were collected and combined according to the similarity. Analytical HPLC was performed on Agilent 1100 apparatus equipped with a diode-array detector and Dionex Ultimate 3000 equipped with UV-Vis detector, respectively. Several Supelco Ascentis Express HPLC columns were used for analysis (RP-Amide, C18, C8, F5, Phenyl, all 10 cm × 2.1 mm, 2.7 μm).

Identification

The isolated compounds were identified using MS and NMR analysis. The MS spectra were obtained via GC-MS analysis of corresponding fractions: fused silica column HP – 5MS 30 m × 0.25 mm coated with film of polymethyl (5% phenyl) siloxane stationary phase. Temperature program: 140 °C hold time 1 min, then increase to 290 °C at a rate 10 °C/min, hold time 16 min. Injector temperature 290 °C. Carrier gas helium, linear velocity 30 cm/sec. Injection volume 1 l, splitter injection, split ratio 1:10. MS conditions: transfer line temperature 280 °C, ion source temperature 200 °C, 70eV, positive ion mode, full scan mode, mass range 50-650 mu. NMR spectra for ^{1}H and ^{13}C analysis were recorded using a Bruker Avance 400 Ultrashield spectrometer operating at a frequency of 400 MHz (^{1}H). NMR spectra were acquired in methanol-d_4 at 298 K and in DMSO-d_6 at 296 K with TMS as an internal standard. The ^{1}H- and ^{13}C-NMR chemical shifts (δ in ppm) were referenced to the signal of the solvent [3.30 ppm (^{1}H) and 49.9 ppm (^{13}C) for methanol-d_4, 2.49 ppm (^{1}H) and 53.6 ppm (^{13}C) for DMSO-d_6]. The 1D NMR experiment and 2D NMR experiments (COSY, HMBC, and HSQC, NOESY and TOCSY) were used to assign the individual ^{1}H and ^{13}C resonances.

2) Results and Discussion

Two groups of chemical compounds were isolated by above described chromatographic procedures from the hexane fractions, and later identified using spectral methods: i/ derivatives of fatty acids: myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, ii/ derivatives of sterols: campesterol, cholesterol, stigmasterol, crinosterol, 7-oxo-sterol.

To isolate other content compounds represented by small molecular substances, we used, similarly to previous experiments, column chromatography, preparative TLC and semipreparative HPLC. TLC on silica was used to choose a mobile phase suitable for separation with sufficient resolution of selected spots. The TLCs were analyzed using UV and visible light or combination of this with detection with sulphuric acid. Extracts and fractions were analyzed using several HPLC methods. We used mobile phase for reversed phase chromatography composed of inorganic solvent (water, formic acid, ammonia) and organic solvent (methanol or acetonitrile) with different stationary phase types (C18, RP-amide, C8). The detection was based on UV, Vis or ELSD detection.

A sequence of methods, which is commonly successful for obtaining small molecular secondary metabolites from natural material, was adopted, but unfortunately without any significant yield of pure substance in amount suitable for the identification and evaluation of biological activity. There are four main hypotheses, which can explain the lack of success of the isolation: 1) in general *P. magnifica* does not produce substantial amounts (below our limits of detection) of secondary metabolites; 2) because the mass of *P. magnifica* is mainly formed from a water rich gel matrix, which is not involved in production of content compounds, the amount of extractible compounds from the material obtained by lyophilization is too low in comparison with extracts obtained typically from natural material (e. g. plants); 3) characteristic compounds present in *P. magnifica* are unstable and conditions used for lyophilization and/or extraction can cause their decomposition; 4) the content compounds produced by *P. magnifica* are of completely different character (e. g. proteins) and thus they are not extractable/detectable by the methods applied.

B. Determination of Cyanobacterial Toxins in P. magnifica by HPLC

Cyanobacteria were found in the *PM* colony gel (typically of genus *Pseudanabaena, Komvophoron, Phormidium, Leptolyngbya*) [17]. Their number increases with the colony lifetime as often indicated by the gel colour.

Microcystins (MCs) and nodularins (NODs), hepatotoxins belonging to a diverse group of cyclic oligopeptides produced by cyanobacteria, where they have been confirmed and determined [18], [19]. Over the last few decades, MCs and NODs have become a serious ecological and health issue due to the massive cyanobacterial water blooms that have developed in eutrophied waters worldwide [18]. The toxicity of and risks from some MC variants have been studied in detail [20], [21], and the World Health Organization recommends a provisional guideline 0.001 mg/L of MC-LR for drinking waters [22]. Therefore, we adapted a published HPLC method [18], [19] in order to monitor these highly toxic compounds.

1) Experimental (Chemical and Methods)

Microcystin-LR, microcystin-RR and NOD were purchased from DHI LAB Products (Hørsholm, Denmark). Acetonitrile, water and methanol of HPLC grade and also formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC system was Dionex Ultimate 3000 (Dionex, USA).

Samples were lyophilized biomass from seasons 2009-2014 collected in Třeboňsko area. Each sample was ultrasonicated

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cork borer. Into the wells 100 μL of cell-free supernatant triplicate were pipette, plates were incubated for 10 hours in 4 °C to let supernatant diffuse to agar, and cultured 24–48 hours at appropriate temperature. Zones of inhibition were measured after cultivation.

B. Results and Discussion

Counts of culturable bacteria in *Pectinatella magnifica* colonies and the surrounding water are listed in Table I. In most cases, all three groups of bacteria were more numerous in the superficial structures of the bryozoan colonies (by one order for aerobic and anaerobic bacteria, in the case of facultative anaerobes by tree orders) compared to gelled mass. More numerous microbiota in superficial layers may be expected because this part contains zooids with simple U-shaped gut that obtains nutrients by water filtration. In both structures of *Pectinatella magnifica* colonies dominated aerobic bacteria with counts one order higher than numbers of facultative anaerobes, and two orders higher than anaerobic bacteria counts. The most variable numbers were obtained after anaerobic cultivation of both structures of *PM* colonies. No trends in the bacterial numbers at each individual locality during each year were observed. It was expected that the highest bacterial counts would be present in colonies sampled from water with the highest microbial contamination, but this was true only in some cases. It was shown that number of microorganisms, in both structures of *Pectinatella magnifica*, did not correlate with the number of microorganisms in surrounding water.

Only one strain of *Pseudomonas moraviensis* 16/12 and two strains of *Aeromonas veronii* 8/12 and 12/13 showed antimicrobial activity against human faecal clostridia. *Aeromonas veronii* 12/13 inhibited also the growth of *Pseudomonas moraviensis* 16/12. Diameters of inhibition zones varied between 8 and 16 mm. Neither of tested conditions nor media affected the antimicrobial activity of tested cell-free supernatants. The growth of other strains was not inhibited by tested bacterial supernatants. Bacteria producing inhibition zones did not decreased pH of cultivation media; so their antimicrobial activity was probably caused by other compounds than acids. Bacteria are well known for the production of many different antibacterial substances. Antimicrobial activity was described for some *Aeromonas* strains [9] and *Pseudomonas fluorescence* is producer of monoxycarbolic acid class antibiotic mupirocin, a mixture of several pseudomonic acids [10], [11]. Mupirocin is effective against aerobic Gram-positive bacteria but most of the anaerobes are resistant [12]. Mupirocin is used for the treatment of skin infections, methicillin resistant *Staphylococcus aureus* and for its activity spectrum is part of cultivation media for the detection of bifidobacteria in fermented and non-fermented milk products containing bifidobacteria and lactic acid bacteria simultaneously (ISO/IDF 220:2009).

IV. Chemical Analysis of Metabolites and Toxins Related to *Pectinatella Magnifica*

Little is known about chemistry of *Pectinatella magnifica*. In 1930 Morse [13] has published preliminary results about composition of the jelly-like secretion of *PM* concluding that the gel is not a collagen-like polymer but rather a true protein which, together with extreme growth rate, he has considered as an interesting case of extremely rapid synthesis of proteins. A course of biuret reaction has been similar to albumins, the gel proteins have been heat-coagulable, and in the proteins the author has confirmed amino acids of tyrosine, tryptophan and cystine. Inorganic compounds of sodium chloride, calcium and, surprisingly, no phosphorus have been confirmed. In statoblasts, the author has presumed presence of common chitin since after hydrolysis glucosamine and galactosamine have been found. Dry mass content of 0.4% has been reported, though our measurements (area Třeboňsko) showed dry mass content up to 2.2% in several samples.

A. Preparative Chromatography and Identification of Content Compounds of Pectinatella magnifica

We worked with lyophilized *PM* collected in Třeboňsko area in seasons 2009-2015 [14]. We focused on content compounds isolated and identified by methods of TLC, HPLC, GC-MS, NMR.

1) Extraction of Lyophilized *Pectinatella magnifica*, Isolation and Identification Process

The extraction procedure was carried out on a large portion of lyophilized *PM*. Firstly, 90% methanol was added to the material. The process of extraction was repeated three times for 24 hours to ensure the quality of extraction. The solvent was each time used fresh. The extract was than filtered and concentrated using rotavapor. The residual water was removed by lyophilization.

The dry extract was dissolved again in 90 % of methanol and 10 % water and was extracted using separatory funnel with hexane in overall ratio 1:1 (v/v, 3×). The combined hexane portions were evaporated to give **hexane** portion of extract. The methanol portion was concentrated using rotavapor to remove large part of methanol. The residue was diluted with water and repeated extraction with chloroform (3 × 24 h) was used to obtain combined **chloroform** portion. The water part was then three times repeatedly extracted with ethylacetate. The combined ethylacetate extracts after removing of solvent on rotavapor yielded **ethylacetate** portion. The water from residue was removed by lyophilization and later **water** portion was yielded.

All fractions obtained in described extraction procedures were analyzed using HPLC and TLC method according to the previously published procedures [15], [16]. No extracts showed significant signal when analyzed using HPLC-DAD detection, we obtained visible spots on TLC only after spraying with sulphuric acid and heating.

Isolation

Hexane and chloroform portions showing the possibility to isolate pure compounds were selected to undergo separation

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for 15 minutes in a water-methanol mixture 1:1 [18], 10 minutes centrifuged and then filtrated (0.45 μm filter) into an HPLC vial.

2) Results

The results of the method transfer are shown in Table II. Typical chromatogram of a mixture of standards is shown in Fig. 2.

TABLE II
EXPERIMENTAL CONDITIONS OF THE ORIGINAL AND TRANSFERRED HPLC METHODS

	Babica *et al.* [18] Blahová *et al.* [19]	This paper
Stationary phase	Supelcosil ABZ+Plus 150x4.6 mm, 5 μm	Ascentis Express RP-amide, 150x2.1 mm, 3 μm
Mobile phase	acetonitrile+TFA*/ water+TFA, flowrate 1.0 mL/min, 30°C	acetonitrile+FA** / water+FA, flowrate 0.5 mL/min, 25°C
Gradient time	30 min	18 min
Detection	UV 238 nm	UV 240 nm
LOD	0.02 mg/kg dry mass	1.3 mg/kg dry mass
Retention time of MCs	11-17 min	3-6 min

* TFA = 0.1% trifluoracetic acid
** FA = 0.1% formic acid

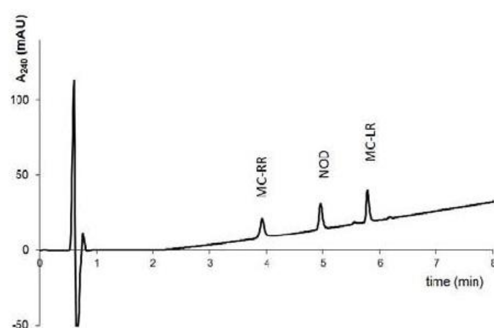

Fig. 2 Chromatogram of a standard mixture of nodularin (NOD) and microcystins RR (MC-RR) and microcystin-LR (MC-LR). The drifting baseline for given wavelength (240 nm) is due to gradient elution. For experimental conditions, see Table II, the rightmost column

3) Estimation of a "Real" LOD on a Model Case of Veselí I

The way of sample preparation (lyophilization of *PM* biomass) means that the calculated LOD relates to a dry mass of *PM*. However, this value does not show a real concentration of the toxins in the water reservoirs.

We tried to estimate LOD in the water (real environment) on a model reservoir with average production of biomass (Veselí I). This number is also a threshold concentration in water that could be determined by the method analyzing dry mass of *PM* after calculation with total water volume of the pond and also to the water volume around the pond bank.

Let us approximate the reservoir with a spherical cap having surface area 240,000 m^2, i.e. periphery of the circle is 1,750 m, radius of 275 m, maximum depth 4.5 m (average depth 3.5 m), and volume of 540,000 m^3. *PM* usually appears within 5 m from the pond bank. Within a strip (transect) of 10 m (i.e. 50 m^2), 30 kg of fresh biomass was collected. Thus, considering the whole periphery, there is 5,250 kg of fresh biomass in the reservoir. If 99% of it is water, we get the amount 52.5 kg of dry mass per 540,000 m^3 of water.

LOD was determined as 1.3 mg/kg of dry matter, which corresponds to 70 mg of toxins per 540,000 m^3, which is equivalent to 0.13 ng/L in water.

We can also estimate a maximum threshold concentration of toxins with the outer circle 5 m from the bank in depth of 1 m (the typical occurrence of *PM*): the volume of water is 8,600 m^3, thus the concentration in water will be 8.2 ng/L.

C. Mupirocin Occurrence in Bacteria Related to *Pectinatella magnifica*

Mupirocin (Bactroban or Centany) is an antibiotic of the monoxycarbolic acid class. It was originally isolated from *Pseudomonas fluorescens* NCIMB 10586 [10]. Mupirocin is bacteriostatic at low concentrations and bactericidal at high concentrations. It is used topically and is effective against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA). It is known that mupirocin is a mixture of several pseudomonic acids [23]. To confirm mupirocin in the samples, an HPLC method [23] was adapted to our conditions.

1) Samples

Supernatants and supernatant dry matters samples were prepared parallely from both *Aeromonas* sp. and *Pseudomonas moraviensis* strains (see *A*, Section III) to be analyzed for presence of bioactive mupirocin. The supernatant dry matter obtained by lyophilization was extracted by methanol. In methanol insoluble portion was treated separately (2x2 samples). The methanol portion was subsequently extracted using separatory funnel to obtain hexane, chloroform, ethyl acetate, and water parts, similarly to *P. magnifica* biomass processing (2x4 samples) (see *A* 1, Section IV).

2) Experimental

Column was Supelcosil LC-8 (Supelco), 5 μm, 150 x 4 mm, mobile phase was acetonitrile with buffer (100 mM ammonium acetate adjusted with acetic acid to pH=4.0), detection @240 nm, flowrate was 1 mL/min, temperature was 25°C. Solvents of HPLC grade, ammonium acetate and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mupirocin was purchased from Applichem (Darmstadt, Germany) No. A4718 (90%).

3) Results

The experimental parameters of the adapted method were optimized as follows: the chromatographic column was Supelcosil LC-8, 150x4 mm, 5 μm; two mobile phases were used – i/ for isocratic elution of the mupirocin standard (70% acetonitrile with 30% of 100 mM ammonium acetate buffer, pH=4.0), ii/ for gradient elution of samples of bacteria extracts (10–60% of acetonitrile in 25 minutes with the same buffer). The flowrate was 1.0 mL/min, temperature was 25°C, UV-detection @240 nm. Estimated LOD was 0.2 mg/L. Applying

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the optimized method isocratic method (run time of 11 minutes, retention time of mupirocin=pseudomonic acid A was 10 min) we confirmed that the commercial substance mupirocin is not a chromatographically pure compound (two extra peaks at 6.5 min and 7.5 min separated with resolution 1.0). In all the samples analyzed, mupirocin was below LOD.

D. Cytotoxicity of Extracts from Pectinatella magnifica

The main aim of the project is to assess potential toxicity of environment invaded by *PM*. Although there is not yet a particular toxic compound related to *PM* occurrence, fractions of lyophilized biomass of *PM* were tested for cytotoxicity.

1) Materials and Methods

Pectinatella magnifica extracts for *in vitro* tests were prepared and supplied by the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic (see *A1*, Section IV). For the purpose of cytotoxicity evaluation, we used THP-1 cell line (European Collection of Cell Cultures, Salisbury, UK), cultured in RPMI 1640 medium (Verviers, Belgium) supplemented with 10% fetal bovine serum, 2% L-glutamine, 1% penicillin and streptomycin at 37 °C with 5% carbon dioxide. All reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Cytotoxicity of *PM* extracts was determined using a LDH assay kit (Roche Diagnostics, Mannheim, Germany) as described previously [24].

For 24 h the THP-1 cells were treated with a various extract concentrations ranging from 10 to 1000 µg/mL in RPMI 1640 medium (concentrations were selected according to [25]).

2) Results

For all tested extracts (PM1 – methanolic extract, PM2 – hexane fraction, PM3 – chloroform fraction, PM4 – ethylacetate fraction, PM5 – aqueous phase), the cytotoxicity was evaluated as a Relative cytotoxicity (relative to control values). Subsequently, from a dose-response curve we derived LD50 values for each of five extracts prepared from *Pectinatella magnifica*. As our results shown, cytotoxicity expressed as LD50 of the following *PM* extracts increased as follows: PM5 (250 ug/mL) > PM2 (75 ug/mL) > PM3 (40 ug/mL) > PM4 (31 ug/mL) > PM1 (29 ug/mL). According to [26] the treatment with *PM* extracts led to toxic effect on THP-1 cells, as their LD50 values were assessed to be <1000 µg/mL.

3) Conclusion

Results from the cytotoxicity assays demonstrated that extracts prepared from *Pectinatella magnifica*, exerted some toxic effect to cells *in vitro*. However, aqueous phase (PM5), which comes into consideration as the most common in nature did not show cytotoxicity. From these results, further studies on *Pectinatella magnifica* should aim its potentially negative effects to the environment.

V. Conclusions

Several points of view were examined related to invasion of *PM* in the area of Třeboňsko. Statistical analysis of *PM* biomass revealed that the invasion of *PM* (spreading to localities) is neither significantly related to a specific locality nor environment. The amount of produced *PM* biomass is very variable among localities and also years (seasons).

PM colonies examined in this study were colonized by bacteria from the environment. However, their abundance was higher than in surrounding water, so their symbiotic relationship with *PM* should not be excluded. Antimicrobial effect of three strains was demonstrated, but the substance responsible for this activity was not identified yet. Related bacterial are known for the production of antibiotic mupirocin, but its presence above LOD in our strains was not proven.

There is no apparent toxicity related to microcystins and nodularin (their concentrations are below LOD of our HPLC method), neither cytotoxicity related to aqueous extracts from *PM*.

We can conclude that the invasion of *PM* in water reservoirs of the Czech Republic does not represent a toxicity risk in this moment. However, a longer-term monitoring is needed.

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5.5. Assessment of chemical impact of invasive bryozoan *Pectinatella magnifica* on the environment: cytotoxicity and antimicrobial activity of *P. magnifica* extracts.

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Assessment of Chemical Impact of Invasive Bryozoan *Pectinatella magnifica* on the Environment: Cytotoxicity and Antimicrobial Activity of *P. magnifica* Extracts

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Abstract: *Pectinatella magnifica*, an invasive bryozoan, might significantly affect ecosystem balance due to its massive occurrence in many areas in Europe and other parts of the world. Biological and chemical analyses are needed to get complete information about the impact of the animal on the environment. In this paper, we aimed to evaluate in vitro cytotoxic effects of five extracts prepared from *P. magnifica* using LDH assay on THP-1 cell line. Antimicrobial activities of extracts against 22 different bacterial strains were tested by microdilution method. Our study showed that all extracts tested, except aqueous portion, demonstrated LD_{50} values below 100 μg/mL, which indicates potential toxicity. The water extract of *P. magnifica* with LD_{50} value of 250 μg/mL also shows potentially harmful effects. Also, an environmental risk resulting from the presence and increasing biomass of potentially toxic benthic cyanobacteria in old colonies should not be underestimated. Toxicity of *Pectinatella* extracts could be partially caused by presence of *Aeromonas* species in material, since we found members of these genera as most abundant bacteria associated with *P. magnifica*. Furthermore, *P. magnifica* seems to be a promising source of certain antimicrobial agents. Its methanolic extract, hexane, and chloroform fractions possessed selective inhibitory effect on some potential pathogens and food spoiling bacteria in the range of MIC 0.5–10 mg/mL. Future effort should be made to isolate and characterize the content compounds derived from *P. magnifica*, which could help to identify the substance(s) responsible for the toxic effects of *P. magnifica* extracts.

Keywords: *Aeromonas*; antimicrobial activity; bacteria; Bryozoa; cyanobacteria; invasive species; *Pectinatella magnifica*; toxicity

1. Introduction

Pectinatella magnifica (Leidy, 1851) is a colonial fresh-water organism from phylum Bryozoa [1], recently invasive in many areas in Europe and other parts of the world

A colony of *P. magnifica* is formed by a layer of zooids, living on a self-produced jelly blob ranging in weight from a few grams to 10 s of kilograms. Similar to other bryozoans, *P. magnifica* is a filter feeders. They feed mainly on micro plankton and detritus [2]. These organisms reproduce, hibernate, and spread through asexual particles, statoblasts. *P. magnifica* is native to the area east of the Mississippi River, from Ontario to Florida. Its first occurrence recorded outside North America was in Western Europe, in Bille River near Hamburg in 1883 (e.g., [3]). During the 20th century, this species gradually spread across the Elbe river into Germany, Czech Republic, and Poland [4,5]. In France, it was recorded occurring in the area called Franche-Comte in 1994 [5,6]. At present, it occurs also in the Netherlands (its occurrence in the Netherlands was first reported in 2003), in the Rhine basin in the area between Luxembourg and Germany, in Austria, Romania and Turkey [7], Hungary [8], and on the island of Corsica [9]. The newest records of presence are published in Japan and the Korean peninsula [10,11].

The spread in slowly flowing streams is certainly significantly conditioned by the water course [5]. Other possible modes could be spreading thanks to zoochory (statoblasts) on feathers of water birds [11], unharmed statoblasts in the content of stomach in some fish species or water birds [12]. Important for spreading can be human activities [13]. The view of Borg [14] is exceptional in that it does not exclude its cosmopolitan origin.

In related marine bryozoans, the specific bioactive compounds, bryostatins, were identified [15]. They primarily have an anticancer effect [16,17]. Bryostatins belong to the class of alkaloids [15]; furthermore, some isoquinolines, sterols, and some carbohydrates with a heteroatom in structure (nitrophenols or disulfides) were also found in bryozoans. Some of them possess the antibacterial and/or cytotoxic activity [18–20]. Except that prevents cell division, some of these metabolites have caused dermatic allergy and have shown antihelmintic activity [21]. Bryostatins are considered to be important promising pharmaceutical substances [17].

Microbial symbionts (e.g., bacteria, cyanobacteria, algae) of bryozoans represent a significant source of potential bioactive compounds [22,23]. For example, bryostatins are produced by the bacterial symbiont *Candidatus Endobugula sertula*, which is present in all life stages of bryozoan *Bugula neritina* [24]. Also, the antimicrobial activity of extracts from marine and freshwater bryozoans including *Pectinatella magnifica* have been demonstrated [18,25–27].

It is assumed that the biomass of *P. magnifica* could contain biologically active substances. Therefore, it is important to study this issue, as well as the composition, the quantity, and activity of microbiota of bryozoan colonies. The main aim of our work was to evaluate in vitro toxicity (Section 2.2) and antimicrobial activity of various extracts prepared from *P. magnifica* (Section 2.3). Further, we analyzed the elementary composition of lyophilized *P. magnifica* gel (Section 2.1) and determined toxins of cyanobacteria related to occurrence of *P. magnifica* (Section 2.4).

2. Results

2.1. Elemental Analysis of P. magnifica Gel

The *P. magnifica* sample for CHN elemental analysis was obtained from a collection of colonies on the pond "Hejtman" in 2014. The gel was mechanically separated from zooids and lyophilized. Elementary analysis showed the composition as 40.0% C, 6.4% H, and 8.7% N.

2.2. *Cytotoxicity of Extracts*

At all five tested extracts, the cytotoxicity was evaluated as a Relative cytotoxicity (Figure 1), relative to control values (vehicle treated groups). Treatment with *P. magnifica* extracts led to significant toxic effects according to [28] (Table 1) on THP-1 cells, as LD_{50} values were assessed to be <1000 μg/mL. Toxicity expressed as LD_{50} derived from a dose-response curve of the following *P. magnifica* extracts increased as follows: PM5 (aqueous portion, 250 μg/mL) > PM2 (hexane portion, 75 μg/mL) > PM3 (chloroform portion, 40 μg/mL) > PM4 (ethyl acetate portion, 31 μg/mL) > PM1 (methanolic extract, 29 μg/mL).

Table 1. Classification of cytotoxicity for natural ingredients [28].

Category	IC_{50}
Potentially very toxic	IC_{50} < 10 μg/mL
Potentially toxic	10 μg/mL < IC_{50} < 100 μg/mL
Potentially harmful	100 μg/mL < IC_{50} < 1000 μg/mL
Potentially non-toxic	IC_{50} >1000 μg/mL

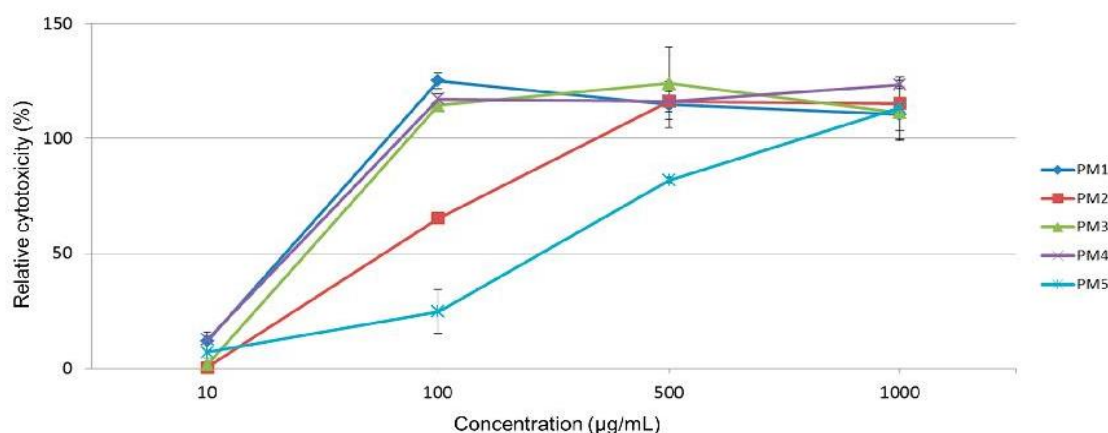

Figure 1. Relative cytotoxicity of *Pectinatella magnifica* extracts in THP-1 cells after 24 h incubation. Legend: PM1—methanolic extract, PM2—hexane portion, PM3—chloroform portion, PM4—ethyl acetate portion, PM5—aqueous portion. The results of LDH release assay are expressed as the means ± SD of three independent experiments, with each condition tested in triplicate.

2.3. *Antimicrobial Activity of Extracts*

Determined MICs of *P. magnifica* extract are showed in Table 2. Only methanolic extract (PM1), hexane (PM2), and chloroform portions (PM3) possessed antibacterial effect against some tested bacteria in the range of MICs from 0.5 to 10 mg/mL. The Gram-positive bacteria were more sensitive to PM1-3 extracts than the Gram-negative. From the Gram-negatives only the growth of *Listeria monocytogenes* ATCC 7644 was inhibited by PM1-3 extracts at MICs 10 mg/mL. The most susceptible bacterium to all three active fractions was potentially pathogenic *Clostridium difficile* CCM 3593. In general, the best results were obtained for chloroform portion (PM3) which inhibits the growth of 10 out of 22 tested strains at the lowest MICs. The hexane portion, inhibiting eight strains, was the second most active substance and methanolic extract affected only four bacterial strains. None of tested bacteria were affected by ethyl acetate and aqueous phase and no growth inhibition caused by DMSO (solvent control) was observed in the control.

Culturable aerobic bacteria were found in the *P. magnifica* colonies in counts of 5.88 ± 0.71 log CFU/g (mean ± S.D., n = 8), the numbers varied between 4.96 and 6.71 log CFU/g. More variable were those bacterial counts obtained after anaerobic cultivation. Viability of anaerobes (including

facultative anaerobes) was from 2.30 to 6.52 CFU/g, 4.12 ± 1.11 log CFU/g in average. Forty isolates out of 49 selected for detailed identification were satisfactorily classified by MALDI-TOF MS analysis. No reliable results were obtained in nine cases. In 40 strains, a secure genus and probable species identification with score values of 2.000–2.299 were observed. *Aeromonas veronii* was found to be the most abundant species (25 strains) in *Pectinatella* colonies, followed by *Aeromonas hydrophila*, *Aeromonas sorbia* (four strains of both species), *Sphingomonas pituitosa*, and *Lactobacillus plantarum* (one strain each). Five strains were identified only to the genus level, two strains as *Chryseobacterium* spp., and two others as *Herbaspirillum* spp., and one as *Pseudomonas* spp.

Our comparison between bryozoan colonies-associated assemblages and those occurring outside bryozoan colonies showed us that cyanobacteria and algae formed a conspicuous biomass mainly in old colonies. When compared with plankton and periphyton, algae, and cyanobacteria demanding a higher trophic degree prevailed (coccal greens *Desmodesmus* spp., *Chlorococcum* etc.; small diatoms *Stephanodiscus hantzschii*, *Nitszchia* cf. *palea*; and filamentous cyanobacteria *Leptolyngbya*, *Komvophoron*, and *Phormidium* spp.).

Table 2. Antimicrobial activities of *P. magnifica* extracts.

Bacterial Strains	Minimal Inhibition Concentrations (mg/mL)		
	PM 1 *	PM 2	PM 3
Micrococcus luteus ATCC 10240	10	4	4
Acinetobacter parvus CCM 7030	>20	>20	>20
Bacillus cereus CCM 2010	5	4	2
Bifidobacterium bifidum DSMZ 20215	10	10	5
Clostridium difficile CCM 3593	1	0.5	0.5
Clostridium perfringens CCM 4435	>20	5	10
Clostridium perfringens DSMZ 11778	>20	5	2
Enterobacter aerogenes CCM 7797	>20	>20	>20
Enterococcus faecalis DMND	>20	>20	>20
Escherichia coli DMND	>20	>20	>20
Escherichia coli O45 IS	>20	>20	>20
Escherichia coli O55 IS	>20	>20	>20
Lactobacillus brevis CCM 3805	>20	>20	>20
Listeria monocytogenes ATCC 7644	10	10	10
Moraxella canis CCM 4590	>20	>20	>20
Propionibacterium acnes DSMZ 1893	>20	>20	>20
Pseudomonas aeruginosa CCM 1960	>20	>20	10
Salmonella enterica Enteritidis ATCC 13076	>20	>20	>20
Salmonella enterica Typhimurium IS	>20	>20	>20
Salmonella sp. DMND	>20	>20	>20
Serratia marcescens DSMZ 30121	>20	>20	>20
Staphylococcus aureus ATCC 25923	>20	10	5

* PM1—methanolic extract, PM2—hexane portion, PM3—chloroform portion. ATTC—American Type Culture Collection, CCM—The Czech Collection of Microorganisms, DMND—The Culture Collection of the Department of Microbiology, Nutrition, and Dietetic of the Life Sciences University Prague, DSMZ—German Resource Centre for Biological Material, IS—The strains provided by Ass. Prof. Ing. Igor Šplíchal, CSc.

2.4. Cyanobacterial Toxins Determination

Cyanobacteria were found in *P. magnifica* colony gels (typically of genus *Pseudanabaena*, *Komvophoron*, *Phormidium*, and *Leptolyngbya*) [29]. Their number increases with the colony lifetime as indicated by the inner colony gel color (from red to green). Toxicity of *P. magnifica* occurrence may come from the cyanobacteria which are known as a source of several hepatotoxins, e.g., microcystins (MCs).

Samples were lyophilized biomass (zooids together with the colony gel) and the surrounding water from the location where the colonies were sampled. The results are summarized in Table 3.

Table 3. Microcystin (MC) determination, the values are concentrations determined in ng/g d.w. for biomass and ng/L for surrounding water.

Location, Sampling Date *	MC–RR	MC–YR	MC–LR	MC–RR	MC–YR	MC–LR
	P. magnifica Colony (ng/g d.w.)			Surrounding Water (ng/L)		
Veselí I, 23.7.2015	31.5	6.6	13.9	1.9	<0.6	3.1
Veselí I, 6.8.2015	6.1	<0.6	10.6	38.3	17.9	70.4
Hejtman, 23.7.2015	4.8	<0.6	7.4	6.8	4.5	31.7
Hejtman, 6.8.2015	4.0	<0.6	2.6	121.6	27.5	159.5
Hejtman, 6.8.2015	11.3	6.1	21.9	n.a.	n.a.	n.a.
Hejtman, 9.10.2012	1.7	<0.6	1.4	n.a.	n.a.	n.a.
Hejtman, 31.10.2012	0.7	<0.6	1.4	n.a.	n.a.	n.a.
Hejtman, 9.10.2012	<0.2	<0.6	<0.2	n.a.	n.a.	n.a.
Hejtman, 9.10.2012	<0.2	<0.6	<0.2	n.a.	n.a.	n.a.

* Location were selected from two monitored places in Třeboňsko area (South Bohemia, CZ): a gravel sandpit (Veselí I) and a pond (Hejtman); MC–RR is microcystin RR, MC–YR is microcystin YR, MC–LR is microcystin LR. Values 0.2, 0.6, and 0.2 ng/g d.w. or ng/L, resp. refers to limit of detections of the respective analytical methods (see 4.4), n.a. stands for "not available".

3. Discussion

Although invasive species are viewed as major threats to ecosystems worldwide, few such species have been studied in detail enough to identify the pathways, magnitudes, and timescales of their impact on native fauna and flora [30]. *P. magnifica* is a new Bryozoa species in Czech Republic with the spread of invasive character [4]. This colonial animal is a filtrator and owing to its massive occurrence it may have an important influence of the ecosystem (species composition, trophic level, hydrochemistry) [31]. This species has not been studied as a key species of any ecosystem yet, and its ecology and chemistry are little known.

Morse [32] has published in 1930 preliminary results about composition of the jelly-like secretion of *P. magnifica* concluding that the gel is not a collagen-like polymer but rather a true protein. Taking into account also an extreme growth rate of the colony gel, he has considered *P. magnifica* as an interesting animal with a rapid proteins production. A course of biuret reaction of the gel has been similar to albumins, the gel has been heat-coagulable, and in the proteins analysis the author has confirmed aminoacids of tyrosine, tryptophan, and cystine. Inorganic compounds of sodium chloride, calcium and, surprisingly, no phosphorus have been confirmed. In statoblasts, the author has presumed the presence of common chitin since, after hydrolysis, glucosamine and galactosamine have been found.

Biomass development in recent seasons 2005–2015 in the Třeboňsko area (South Bohemia, Czech Republic) and monitoring of MC–RR, MC–LR, and nodularins (an HPLC method with the limit of detection of 0.2 mg/L) were already reported [33].

3.1. Elemental Analysis of P. magnifica Gel

P. magnifica forms huge jelly colonies, composed from material, which has still not been chemically well elucidated. Although the name "Pectinatella" resembles pectin molecules as basic building blocks, it seems that the name more probably comes from the physico-chemical properties of the jelly colony. The CHN elementary composition (40.0% C, 6.4% H, and 8.7% N) shows, that the analyzed material is a mixed composition of protein and polysaccharide, or a strongly glycosylated protein, as pure proteins usually show twice higher content of nitrogen. It can be speculated that the compound(s) that form the colony gel should be relatively simple compound(s) considering the zooid/colony size (2–4 mm/200–400 mm), sources of nutrition (fresh-water), and the enormous growth rate ($\approx$0.5 m in diameter/month).

On the other hand, the analyzed material could be partially contaminated by presence of bacteria, cyanobacteria, and algae, as described further. Chemical composition of the colony gel and the zooid layer is under examination.

3.2. Cytotoxicity Tests

Since in related marine bryozoans (phylum Bryozoa) the specific bioactive or toxic compounds were identified [15], we aimed to evaluate cytotoxic effects of the *P. magnifica* lyophilized material. Based on our previously published results [34,35] we used THP-1 cell line as a model system to detect cytotoxic effects of extracts prepared from *P. magnifica*. Results of the toxicity assay showed us that all samples of evaluated *P. magnifica* extracts—except aqueous phase—demonstrated LD_{50} values below 100 µg/mL, which indicate to potential toxicity. The water extract of *P. magnifica* with LD_{50} value of 250 µg/mL also shows potentially harmful effects. Since the behavior of *Pectinatella* exerts signs of invasive character, its potential direct or environmental toxicity should be further studied. However, the tests carried out by our group are using the leukemic THP-1 cells and more test on normal non-cancer cells should be carried out to verify or to refute the toxicity.

3.3. Antimicrobial Activity

Extracts of marine [18] and other species of freshwater bryozoans [25] including *P. magnifica* [26,27] have previously been tested to determine their antimicrobial activities. In correspondence with the need of discovering new potentially antimicrobial natural products, bacteria inhibiting activity of *P. magnifica* extracts described in this study, may be considered as promising. The highest antibacterial effect of *P. magnifica* methanolic, hexane, and chloroform extracts was observed against *Clostridium difficile*, bacterium known as the pathogen responsible for various intestinal disorders [36]. Also, other potential pathogens (*Clostridium perfringens*, *Staphylococcus aureus*) and food spoiling bacteria (*Bacillus cereus*, *Listeria monocytogenes*) were inhibited by tested extracts. Recently also Pejin et al. [26] determined antimicrobial activity of crude extracts of *P. magnifica*. In contrast to our experiment, tested bacteria were inhibited not only by methanolic, hexane, and chloroform extract but also by ether and aqueous fractions. *Salmonella*, *Escherichia*, and enterococci tested in our study were resistant to *P. magnifica* extracts whilst Pejin et al. [26] demonstrated effect against these bacteria. *S. aureus*, *B. cereus*, *Micrococcus* spp., *L. monocytogenes*, and *Pseudomonas aeruginosa* were sensitive to *Pectinatella* extract in both studies but MIC determined in our experiment were much higher (2–10 mg/mL) compared to Pejin et al. [26], who demonstrated MIC lower than 1 mg/mL in all cases. The differences in MICs values could be caused by different content of active compounds in *Pectinatella* extract due to dissimilar microbial colonization, environment composition, and methods of extract preparation. Similarly to our experiments, Pejin et al. [25] described inhibition effect of hexane fraction from freshwater *Hyalinella punctata* against *B. cereus*, *L. monocytogenes*, and *S. aureus*. In contradiction with our results, hexane fraction from *Hyalinella punctata* was active also against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica Typhimurium*. Antimicrobial activity of extracts from different species of Antarctic bryozoans against *B. cereus* was also reported [18], but at tested ether extracts. Although, MICs determined in our experiments were relatively high—much higher than those usual for antibiotic—it must be taken in account that the mixtures of substances were tested. Further, methanolic extract, hexane, and chloroform portions from *P. magnifica* will be separated, identified, and tested for the antimicrobial activity.

Because contamination with foreign bacteria from surrounding water could not be completely prevented during the sampling for microbiological analysis, an important caveat is that not all cultured bacteria may be host specific or even symbiotic. Identified bacteria had previously been described as ubiquitous in water, soil, plants, and other environmental samples. Zooids situated on the surface of *Pectinatella* colonies possess simple U-shaped gut which is presumably inhabited by described bacteria. We can only speculate, if these genera are part of natural gut microbiota or may be pathogenic for *Pectinatella*. *Aeromonas veronii*, the most frequently isolated bacteria, is species ubiquitous in fresh water and have been found in association with a variety of vertebrates and invertebrates with both beneficial and pathogenic outcomes. They have been reported to cause wound infections and diarrhea in humans and have been found as a pathogen and a gut symbiont in some fishes [37]. Sreedharan et al. [38] demonstrated marked cytotoxic and hemolytic activity, which were responsible for the pathogenic

potential of *Ae. veronii* strains. This information may indicate that bacteria colonizing *Pectinatella* may be liable for the toxicity of its extracts. Since, simultaneous cytotoxic and antibacterial activity is common for many substances produced by microorganisms, bacteria associated with bryozoan colonies may be responsible also for the antimicrobial activity of their extracts [39].

Toxicity caused by planktonic cyanobacteria such as *Microcystis* and *Woronichinia* does not appear to be an issue, because of a relatively scarce abundance of these planktonic species in matrix. Nevertheless, an enrichment in the rising biomass of benthic filamentous cyanobacteria (*Leptolyngbya*, *Komvophoron*, less also *Phormidium*), compared with surrounding water, can bring a significant dose of cyanotoxins (produced by them) to environment mainly during decomposition of *Pectinatella* colonies. All these genera include a plethora of species, many of which are known to produce toxins including microcystins (MCs) [40,41]. Benthic cyanobacterial assemblages are commonly formed by a mixture of toxic and non-toxic genotypes and toxin concentrations can be highly variable in space and time [42]. Even in a very small sample (such as 1 cm^2) of a benthic mat formed by *Phormidium* can coexist both toxic (anatoxins producing) and non-toxic strains [31]. However, at present, it is not possible to distinguish between two plausible mechanisms (direct toxic effect of *P. magnifica* vs. toxicity of symbiotic bacteria) on the basis of the data in this study. Thus, in the nearest future, we put our efforts into the isolation and characterization of content compounds derived from *P. magnifica*, which could help us to identify the substance(s) responsible for the toxic effects of *P. magnifica* extracts.

3.4. Cyanobacterial Toxins Determination

Microcystins (MCs) belong to a diverse group of oligopeptides produced by cyanobacteria. Over the last few decades, MC and nodularins have become a serious ecological and health issue due to the massive cyanobacterial water blooms that have developed in eutrophied waters worldwide [43].

The toxicity of and risks from some MC variants have been studied in detail [44,45], and the World Health Organization recommends a provisional guideline 1 μg/L of MC–LR for drinking water [46]. Therefore, we adapted a published LC-MS/MS method [47,48] in order to monitor the most common structural (MC–RR, MC–YR, and MC–LR) variants of these highly toxic compounds.

Cyanobacteria and algae were found both in *P. magnifica* matrix and inner surface (a part of a colony attached to substrate). They can play an important role in production of bioactive compounds [29]. Assemblages associated with bryozoan colonies were compared with those occurring outside bryozoan colonies. Several deductions can be made from data in Table 3: (a) three of the four samples (lines 2–4) exhibit much lower concentrations of toxins in the colony than in the surrounding water, which excludes a significant effect of pre-concentration of cyanobacteria inside the colony; (b) the pond "Hejtman" exhibits higher concentration of MCs than the gravel sandpit "Veselí I", which can be explained by higher trophicity of pond waters; and (c) values in August exceed those in July which follows a natural growth of the animal biomass.

MC concentrations (sum of the three major structural variants) in the surrounding water of the two sampling sites ranged between 5–309 ng/L, which is below the WHO limit (1 μg/L), and also relatively lower than the typical concentrations found in the Czech Republic or other countries [49,50]. For example, median concentration of MCs in water samples collected in Czech reservoirs during the summer of 2012 was 970 ng/L, and concentration of microcystins exceeded 300 ng/L in 70% samples [50]. Concentrations of MCs detected in the biomass of *Pectinatella* ranged between 2–52 ng/g d.w., were lower than concentrations typically present in cyanobacterial water blooms, where MC concentrations can reach several mg/g d.w. [49]. However, MCs can be often detected in the environment and biota samples also in ng/g d.w. levels, as reported for cyanobacterium *Gloeotrichia* collected from oligotrophic reservoirs [51], cyanobacteria *Leptolyngbya* and *Geitlerinema* isolated from coral reefs [52]. Similarly, trace concentrations of MCs could be found also in lichen [53] or periphyton samples [54], tissues of aquatic organisms exposed to MCs [55,56], or in freshwater sediments [43].

4. Materials and Methods

4.1. Elemental Analysis of *P. magnifica* Gel

The lyophilized gel was used for determination of elementary composition. The PE 2400 Series II CHNS/O Analyzer (Perkin Elmer, Waltham, MA, USA) was used for simultaneous determination of C, H, and N in the sample. In the CHN operating mode the instrument employs a classical combustion principle to convert the sample elements to simple gases (CO_2, H_2O, and N_2). The PE 2400 analyzer performs automatically combustion and reduction, homogenization of product gases, separation, and detection. A microbalance MX5 (Mettler Toledo, Greifensee, Switzerland) was used for precise weighing of samples.

4.2. Cytotoxicity Tests

The biomass of *P. magnifica* was obtained on July and August 2012 at selected locations in Třeboňsko basin (South Bohemia, Czech Republic), samples were rapidly frozen at −30 °C and then lyophilized. Extracts from *P. magnifica* lyophilisate were prepared in the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. Lyophilized *P. magnifica* biomass (980 g) was extracted in 70% methanol (3 × 24 h, PM1). The extract was dissolved in 90% methanol and extracted with hexane (3 × 1 L) to get (after removal of hexane by rotavapor) hexane portion (15 g, PM2). The methanol part was diluted with water to 20% methanol solution and extracted with chloroform (3 × 1 L). Chloroform was removed from chloroform portion using rotavapor to get 3 g of PM3. The water residue was consequently extracted with ethylacetate (3 × 1 L) to obtain (after removal of ethylacetate using rotavapor) 10 g of PM4. The water from the water portion was removed via lyophilization to yield 26 g of PM5.

RPMI 1640 culture media, phosphate buffered saline (PBS), and antibiotics (penicillin and streptomycin) were purchased from Lonza (Verviers, Belgium). Fetal bovine serum (FBS) was purchased from (PAA Laboratories, Pasching, Austria). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

The human THP-1 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK; Methods of characterization: DNA Fingerprinting (Multilocus probes) and isoenzyme analysis). Cells were cultured in RPMI 1640 medium supplemented with antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin), 10% FBS, and 2 mM L-glutamine. Cultures were kept in an incubator at 37 °C in a water-saturated 5% CO_2 atmosphere in air. Cells were passaged at approximately one-week intervals. Cells were free from mycoplasma infection (Hoechst 33258 staining method).

Cytotoxicity of *P. magnifica* extracts was determined using a LDH assay kit (Roche Diagnostics, Mannheim, Germany) as described previously [34]. THP-1 cells were exposed for 24 h at 37 °C to various extract concentrations ranging from 10 to 1000 µg/mL in RPMI 1640 medium (concentrations were selected according to [57]. For LDH assays, cells were seeded into 96-well plates (5×10^4 cells/well in 100 µL culture medium) in triplicate in serum-free RPMI 1640 medium and measurements were taken 24 h after the treatment with the compounds. The median lethal dose values, LD_{50}, were deduced through the production of a dose-response curve. The maximum concentration of DMSO in the assays never exceeded 0.1%. All data from three independent experiments were evaluated using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

4.3. Antimicrobial Activity

All five *P. magnifica* extracts were tested for their antimicrobial activity against bacteria listed in Table 1. Tested bacteria (potential pathogens, food-spoiling psychrophilic bacteria, and intestinal bacteria) were subcultured in appropriate media and conditions for 24 h prior to the test. A broth microdilution method was used to determine minimal inhibition concentrations (MICs) of the PM1-5 extracts. All the *P. magnifica* extracts were diluted in dimethyl sulfoxide (DMSO), its concentration did not exceed 1% (a concentration inhibiting bacterial growth). Two-fold dilutions were carried

out, starting from an initial concentration of 20 mg/mL, employing 96-well microtiter plates. The bacterial inoculum was standardized to achieve density of 1×10^6 CFU/mL. For the anaerobes (clostridia and bifidobacteria), the plates were prepared in an anaerobic chamber (Bugbox, BioTrace, Bridgend, UK) and then incubated for 48 h at 37 °C in an anaerobic jar (Anaerobic Plus System, Oxoid, Basingstoke, UK). Plates were prepared and cultivated under aerobic condition for the rest of tested bacteria. The turbidity in the wells was determined spectrophotometrically using a Microplate reader Infinite M200 (Tecan Trading AG, Männedorf, Switzerland) at 420 nm to evaluate the growth. MICs were defined as the lowest concentration of a *P. magnifica* extract inhibiting growth of the tested bacteria by ≥80% compared to the control. The tests were done in triplicates.

Eight *Pectinatella* samples for microbiota analysis were aseptically collected, transferred to vials containing oxygen-free peptone water, kept in a refrigerator and analyzed within five hours after collection. Samples were serially diluted in the Wilkins-Chalgren broth (Oxoid, Basingstoke, UK) under anaerobic condition. Appropriate dilutions were transferred to Petri dishes and immediately filled with yeast extract-tryptone agar (YT; Oxoid) supplemented with 1 g/L of glucose. Plates were incubated under both anaerobic condition (Anaerobic Plus System; Oxoid) at 25 °C for five days and aerobic condition at the same temperature for three days.

After incubation, six colonies representing a wide range of pigments and colony types were picked up from each sample and cultivation variant. Bacteria were enriched in YT medium and identified. A total of 96 isolates were evaluated for their purity and morphological characteristics using phase contrast microscopy and Gram staining. Forty-nine pure strains with different morphologies were used for detailed identification by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS, Bruker Daltonik GmbH, Leipzig, Germany) using the MALDI BioTyper (TM) system (Bruker Daltonik GmbH, Germany) according to [58]. The obtained raw spectra were analyzed using BioTyper software (version 2.0, Bruker Daltonik GmbH, Leipzig, Germany).

4.4. Cyanobacterial Toxins Determination

4.4.1. Sample Preparation

A sample of water (500 mL) was lyophilized, the residues dissolved in 1 mL of 50% methanol/water, then vortexed for 1 min, transferred into an Eppendorf vial, and finally centrifuged (10 min at 21,255× *g*). The supernatant was collected, filtrated (0.45 μm nylon filter) into an HPLC vial, and directly analyzed. Lyophilized biomass was weighed (cca 0.1 g) and transferred into a 15 mL centrifuge vial.

A biomass extraction step started with addition of 3 mL of methanol to 0.1 mg of freeze-dried biomass. The mixture was vortexed for 2 min, sonicated by a sonication needle (Bandelin Sonopuls HD2070 with MS72 probe, 3 × 20 s, cycle 0.9 × 10%, power 70%–75%) and then centrifuged (15 min at 3095× *g*). The supernatant was taken and filtrated (0.45 μm nylon filter) into a 12 mL glass vial. Then the whole process of extraction was repeated (starting with addition of 3 mL of methanol) and the second supernatant portion was combined with the first one. Then the mixture was diluted with 110 mL of MilliQ water (the organic phase ratio approximately 5%).

A solid phase extraction column (Oasis HLB, 60 mg) was first conditioned with 2 mL of methanol by the means of the vacuum manifold, and then equilibrated with 2 mL of MilliQ water. The biomass extract prepared according to previous steps was applied onto the column. Prior the elution, the column was washed with 2 mL of 20% methanol, and the analytes were finally eluted with 2 mL of methanol. Then the solvent of the extract was evaporated to dryness under a nitrogen flow at 45 °C. Finally, the sample was diluted with 200 μL of 50% methanol and vortexed for 1 min, transferred into an Eppendorf vial, and centrifuged (10 min at 21,255× *g*). The supernatant was filtrated (0.45 μm nylon filter) and transferred into an HPLC vial.

4.4.2. MC Determination

The analytical method was based on a protocol suitable for MC detection in very complex matrices such as animal tissues [47]. An Agilent 1260 Infinity liquid chromatograph was used with quaternary pump, thermostatted autosampler, and column oven (Agilent Technologies Inc., Waldbronn, Germany). The analytical column was an Agilent POROSHELL 120 EC-C18 (Agilent Technologies Inc., Santa Clara, CA, USA) (4.6 × 50 mm, 2.7 μm) and Guard column (2.1 × 5 mm, 2.7 μm). The injection volume was 5 μL and the flow rate was set to 0.35 mL/min. The mobile phase (A) was 0.1% formic acid in water, and the mobile phase (B) was 0.1% formic acid in acetonitrile. Gradient conditions were 70% of (A), ramped to 45% (A) over 5 min, then decreased to 10% of (A) from 5.01 and held until 7.0 min. Under these conditions, the retention time was 3.5 min for MC–RR, 4.7 and 4.9 min for MC–YR and MC–LR, respectively.

An Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies Inc.) with an electrospray ionization (ESI) source was used in positive mode: a nitrogen gas temperature 300 °C and gas flow 5 L/min, nebulizer pressure 45 psi, sheath gas temperature 350 °C and flow 11 L/min, capillary voltage 4000 V, and nozzle voltage 500 V. The dwell time of each compound was 200 ms. Transition *m/z* monitored in LC-MS/MS with multiple reaction monitoring (MRM) mode, collision (CE) and fragmentation energy (FE): MC–RR 520 → 135 (CE 60, FE 203), MC–YR 1046 → 135 (CE 61, FE 130), and MC–LR 996 → 135 (CE 68, FE 206). Matrix effect was compensated by external sample calibration.

Instrumental limit of detection was 0.1 ng/mL, 0.3 ng/mL, and 0.1 ng/mL for MC–RR, MC–YR, and MC–LR resp., which corresponds to method limit of detections 0.2 (MC–RR), 0.6 (MC–YR), and 0.2 (MC–LR) ng/g d.w. or ng/L.

5. Conclusions

Since a typical elemental composition of proteins is 56% (carbon), 7% (hydrogen), and 14% (nitrogen) resp., the hypothesis of Morse [32] about true protein composition of PM gel can be falsified. Lower content of carbon and nitrogen found (40.0% and 8.7% resp.) rather suggests admixture of carbohydrates that typically exhibit 40% of carbon (and no nitrogen), so in the PM gel we can presume presence of highly glycosylated protein(s). Also, composition of PM biomass was studied earlier [33]. Two types of chemical compounds were isolated identified from *P. magnifica*: (a) derivatives of fatty acids: myristic acid, pentadecanoic acid, palmitic acid, margaric acid, and stearic acid; and (b) derivatives of sterols: campesterol, cholesterol, stigmasterol, crinosterol, and 7-oxo-sterol.

Hepatotoxic peptides of cyanobacterial origin (MCs) were found in *P. magnifica* samples, but only at levels not exceeding the concentrations typically found in aquatic biota. However, only trace concentrations of MCs were present in the surrounding water, and their levels did not correlate with MC content in bryozoan samples. Thus, cyanobacteria present in *Pectinatella* colonies might be the source of MCs detected in the biomass, rather than contamination by residues of the surrounding water during the sampling.

We have also been monitoring MC content in PM biomass for the last years with a HPLC method reported previously [33]. However, the method employs a UV detector—i.e., it exhibits a higher LOD = 1.3 mg/kg d.w. On the other hand, this LOD in dry biomass corresponds to a real concentration of MC in water 0.13 ng/L [33]. Until now we have observed no sample that would overstep the limit. Our initial findings of MCs in the biomass of *P. magnifica* indicated probably for the first-time possible biosynthesis of hazardous cyanotoxins by cyanobacterial species colonizing the bryozoan biomass. Further research should focus on isolation and identification of cyanotoxin-producing species and strains, as well as on detailed characterization of seasonal and spatial dynamics of MCs concentration in the bryozoans and in the surrounding water. This would provide not only information relevant for assessment of ecological and human health risks of bryozoan-associated cyanotoxin contamination, but possibly contribute also to our understanding of the ecophysiological role of cyanotoxin production, its impact on the host species or its eventual role in the host-guest chemical interactions.

Based on the results from Sections 2.2 and 2.3, it can be concluded that all tested *P. magnifica* extracts, except PM5 (aqueous phase), can be considered as potentially toxic. Methanolic (PM1) and ethyl acetate (PM4) extracts showed the highest cytotoxicity; conversely aqueous phase may be regarded as potentially harmful. From these results, further studies on *P. magnifica* should aim its potential negative effects to the environment. It may be assumed that cytotoxic compounds detected in *Pectinatella* extracts could be produced by *Aeromonas*, since the members of these genera were found as the most abundant bacteria associated with *P. magnifica*.

Although, MICs of *P. magnifica* extracts determined in our experiments were relatively high, bacteria inhibiting activity, may be considered as promising, because the mixtures of substances were tested. Further, methanolic extract, hexane, and chloroform portions from *P. magnifica* could be identified and tested for the antimicrobial activity.

An environmental risk resulting from the presence and increasing biomass of potentially toxic benthic cyanobacteria in old colonies should not be underestimated. On the other hand, since the methanolic extract, hexane, and chloroform fractions possessed selective antibacterial effects, *P. magnifica* may be considered as a promising source of antimicrobial substances.

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Author Contributions: P.K. performed the literature search, wrote the manuscript, and was responsible for cytotoxic studies. K.S. wrote the manuscript and performed extractions. H.S. and E.V. wrote manuscript and performed antibacterial activity assays and microbial analyses. O.L.-S. performed the analysis of cyanobacteria and algae. Z.B. and J.R. were responsible for P. *magnifica* harvest and identification. J.S. was responsible for elementary analysis. J.C. was responsible for the elemental analysis. L.J. and P.B carried out the microcystins determination. J.P. revised the manuscript and coordinated the overall work.

Conflicts of Interest: The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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6. Sumární diskuse

Existuje celá řada přístupů ke studiu mikroorganismů, přičemž každý s sebou přináší určité výhody a omezení (Článek 1). Co se týká stanovení skutečné bakteriální diversity, není pochyb o tom, že nejpřesnější informace jsou získávány pomocí moderních sekvenačních metod (Schuster 2008). To platí zejména pro mikroorganismy, které nejsou schopné růstu v experimentálních podmínkách, jsou v prostředí zastoupené menšinově nebo jsou pomalu rostoucí. Na druhou stranu, izolace bakterií v axenické kultuře je prvním krokem k určení nových taxonů. Kultivace umožňuje studium celého organismu, včetně fenotypových charakteristik, což mimo jiné umožňuje ověřit hypotézy vyvstávající z genomických dat. Také kompletní genomy o vysoké kvalitě, které slouží jako referenční pro řadu meta-omických technik, a pro tvorbu specifických sond a primerů, jsou získávány z axenických kultur. Význam a potřebu izolace vodních mikroorganismů ve svých pracích zdůrazňují např. Giovannoni and Stingl 2007; Garcia 2016 nebo Salcher and Šimek 2016. Aby bylo možné mikroorganismy z *Pectinatella magnifica* blíže charakterizovat, studovat jejich metabolický potenciál a případnou biologickou aktivitu, byla tato práce zaměřena na kultivovatelnou frakci mikrobioty této mechovky.

Prvním cílem doktorské disertační práce bylo izolovat a identifikovat bakterie z bochnatky americké (Článek 2, Článek 3). Pro stanovení počtů kultivovatelných bakterií a jejich následnou izolaci byla použita dvě kultivační média, již dříve popsaná pro mořské druhy (González et al. 1996), s modifikací pro sladkovodní prostředí. Celkové počty, stanovené na těchto médiích, se vzájemně statisticky významně nelišily. V povrchové vrstvě kolonií *P. magnifica* bylo zjištěno statisticky významně vyšší množství mikroorganismů, než ve vnitřní gelové hmotě. V obou strukturách převládaly aerobní bakterie, v povrchové vrstvě společně s fakultativně anaerobními. Nejméně zastoupeny byly bakterie striktně anaerobní, které byly odlišené od fakultativních anaerobů přídavkem neomycinu do kultivačního média, o koncentraci 70 mg/l. Neomycin by měl v této koncentraci růst fakultativních anaerobů inhibovat (Pinheiro et al. 2003). Nebyly zjištěny žádné závislosti mezi hodnotami z jednotlivých let, lokalit ani odběrů. Celkové počty byly studovány také kultivačně nezávislou metodou fluorescenční *in situ* hybridizace (FISH) a vyhodnocovány mikroskopicky. Tento přístup se ukázal jako nevhodný pro daný typ vzorků z důvodu vysokého detekčního limitu, který se uvádí 10^6 KTJ/g (ml) (McCartney 2002). Hodnoty stanovené kultivačně se pohybovaly v rozmezí od 10^3 do 10^9 KTJ/g. Vyhodnocení vzorků s vyššími počty bakterií bylo znesnadněno nespecifickým navázáním fluorescenční sondy na materiál samotného vzorku.

Izoláty bakterií byly identifikovány pomocí MALDI-TOF hmotnostní spektrometrie a sekvenace genu pro 16S rRNA (Weisburg et al. 1991), Sangerovou metodou (GATC Biotech). Jako nástroj pro studium genetické variability v rámci jednotlivých fylogenetických skupin, a eliminaci genomicky identických kmenů pro identifikaci, byla také použita fingerprintová metoda REP-PCR (Gevers et al. 2001). V koloniích mechovky zcela jasně dominovali zástupci Gammaproteobacteria (n = 104), následovaní Betaproteobacteria (n = 36), Firmicutes (n = 11), Bacteroidetes (n = 6) a nejméně početnou skupinou, Alphaproteobacteria (n = 1). V rámci Gammaproteobacteria, patřilo 87 % izolovaných kmenů do rodu *Aeromonas*. Dále byli detekováni zástupci rodů *Acinetobacter*, *Enterobacter*, *Klebsiella*, *Lelliottia*, *Plesiomonas*, *Pseudomonas* a *Shewanella.* Třída Betaproteobacteria byla zastoupena 5 rody: *Aquitalea*, *Herbaspirillum*, *Chromobacterium*, *Paludibacterium* a *Rhodoferax*. Firmicutes reprezentovaly rody *Bacillus*, *Clostridium*, *Lactococcus* a *Leuconostoc*, a dva nepopsané rody v rámci čeledí *Clostridiaceae* a *Sporomusaceae*. V rámci Bacteroidetes byly nalezeny rody *Chryseobacterium* a *Chitinophaga*, a u Alphaproteobacteria jako jediný, rod *Sphingomonas*. V rámci naší studie (Článek 3) bylo nalezeno celkem 18 bakteriálních fylotypů, které představují 15 potenciálně nových druhů příslušných rodům *Aeromonas*, *Aquitalea*, *Clostridium*, *Herbaspirillum*, *Chromobacterium*, *Chryseobacterium*, *Morganella*, *Paludibacterium*, *Pectobacterium*, *Rahnella*, *Rhodoferax* a *Serratia*, a 3 potenciálně nové rody patřící do čeledí *Clostridiaceae* a *Sporomusaceae*. Většina z nich byla izolována z kolonií mechovky, ostatní pak z okolní vody. *Clostridiaceae bacterium* PT50 byl, jako jediný potenciálně nový taxon, nalezený v obou typech vzorků. V době, kdy se začínala zpracovávat tato disertační práce, nebyly dostupné žádné publikace, zabývající se mikrobiotou osidlující sladkovodní mechovky nebo jejich vzájemnými specifickými interakcemi. Mikrobiální diversita kultivovatelných bakterií byla studována u několika mořských druhů (Pukall et al. 2001, Heindl et al. 2010). Porovnání našich výsledů s výsledky studií, zaměřených na mořské formy však není zcela relevantní, protože bakterie vyskytující se ve sladké vodě jsou od mořských často evolučně vzdálené (Zwart et al. 2002). Nicméně, Pukall *et al.* (2001) došli k závěru, že povrch mechovky *Flustra foliacea* je osidlován bakteriemi, které jsou zcela běžné pro mořské prostředí a/nebo byly do tohoto prostředí zavlečené z pevniny, což je v souladu s našimi výsledky. Většina bakterií, které byly izolované z *P. magnifica*, jsou druhy běžně se vyskytující ve vodě a/nebo životním prostředí (Arai et al. 1980, Sugita et al. 1995, Quadt-Hallmann & Kloepper 1996, Stock & Wiedemann 2002, Lau et al. 2006, Herzog et al. 2008, Han et al. 2008, Weon et al. 2009, Adav et al. 2010, Huang et al. 2010, Ramette et al. 2011, Wisplinghoff et al. 2012, Tang et al. 2012, Monteiro et

al. 2012, Bais et al. 2013, Kämpfer et al. 2014, Yadav et al. 2015, Ng & Sudesh 2016, Sasi Jyothsna et al. 2016, Austin & Austin 2016).

Nejvyšší počty bakterií byly očekávány u vzorů kolonií odebraných z vody s nejvyšší mikrobiální kontaminací. To však bylo potvrzeno pouze v některých případech. Statistická analýza prokázala, že množství bakterií přítomných v koloniích mechovky není závislá na množství bakterií v okolní vodě. Toto zjištění poukazuje na komensální či symbiotický charakter těchto organismů. Specifické bakteriální komunity bývají silně závislé na charakteristických vlastnostech svého hostitele, které je někdy velmi obtížné, ne-li nemožné, napodobit v podmínkách *in vitro*. Aby byl růst specifických bakterií podpořen, byl do kultivačního média přidán extrakt z *P. magnifica*. Většina bakterií patřící mezi Betaproteobacteria byla izolovaná výhradně z kolonií mechovky, přičemž 7 ze 13 detekovaných druhů představuje potenciálně nové taxony. Lze tedy předpokládat, že bakterie žijící v symbióze s *P. magnifica* patří právě do této skupiny. Tento předpoklad je podpořen faktem, že 7 kmenů *Herbaspirillum* sp. PT3, představující potenciálně nový druh, bylo nalezeno v koloniích mechovky ze všech lokalit, ale žádný nebyl detekován ve vzorcích vody. Sekvence 16S rDNA tohoto druhu je shodná se sekvencí *Oxalobacteraceae* bacterium PRE7E (KM187634), izolované z kůže čolka zelenavého (*Notophthalmus viridescens*). Obdobným případem je *Aquitalea* spp. PT67 a PT99, které byly podobné sekvencím dosud nepopsaných druhů čeledi *Neisseriaceae*, izolovaných z kůže skokana volského (*Rana catesbeiana*) (PT67 - FE64E [KM187092] a PT99 - BFE40G [KM187049]). Oba obojživelníci pocházejí, stejně jako *P. magnifica*, ze Severní Ameriky, a všechny 3 s nimi spojené izoláty byly získány ze vzorků v Severní Americe v rámci jedné studie (Walke et al. 2015). Vyvstává tedy otázka, zda mohly být tyto bakterie distribuovány společně s mechovkou, což by opět naznačovalo jejich možné vzájemné interakce. Druh *Vibrio fisheri*, který je často uváděn jako modelový organismus pro studium iniciace, osídlení a přetrvání symbiózy, se vyskytuje volně ve vodě. Je tedy detekovatelný i na jiných mořských makroorganismech. Až při kontaktu s cílovým hostitelem (*Euprymna scolopes*) osidluje *V. fisherii* jeho světelný orgán, kde se hojně pomnoží díky poskytovaným živinám, a vzniká vzájemný mutualistický vztah (Lupp & Ruby 2005). Jedná se o horizontální přenos, kterým jsou charakteristické sekundární symbiotické mikroorganismy. Ty po kolonizaci naivního hostitele mohou být dále přenášeni vertikálně (Dale & Moran 2006). Tímto způsobem by teoreticky mohly být distribuovány i výše zmíněné druhy, izolované z *P. magnifica*, jejíž kolonie taktéž představují niku s vyšší úrovní trofizace než okolní prostředí (Lacourt 1968).

Diskutabilní je specifický vztah nových druhů rodu *Paludibacterium*, PT78 a PT71. Ačkoliv nebyly dosud validně popsány, jejich sekvence 16S rDNA jsou téměř shodné se sekvencemi kmenů, nalezených v terestriálním prostředí. PT78 je blízce příbuzný s *Paludibacterium* sp. J8SN6 (JF327660) detekovaném v půdě a PT71 s *Paludibacterium* sp. CRh31 (KR780468) izolovaném z divoké rýže. Přestože sekvence nebyly zcela identické a specifická adaptace (Yi et al. 2017) nemůže být vyloučena, jedná se spíše o organismy, které se do kolonií dostaly z okolního prostředí, než o hostitelsky specifické symbionty (Kwon et al. 2008, Sheu et al. 2014, Kang et al. 2016). To platí také pro *Rhodoferax* sp. PT111 a *Chryseobacterium* sp. PT238 (Zwart et al. 2002, Herzog et al. 2008, Kämpfer et al. 2014). Ze všech kmenů, které byly izolované v rámci této práce, se nejpravděpodobnějším symbiotickým mikroorganismem *P. magnifica* zdá být druh PT79, taktéž ze skupiny Betaproteobacteria, klasifikovaný jako *Chromobacterium* sp. Symbiotické mikroorganismy mořských mechovek bývají účastny chemické obrany svého hostitele skrze produkci biologicky aktivních metabolitů. Za nejvýznamnější vlastnosti těchto látek je považována antimikrobiální a/nebo cytotoxická aktivita (Sharp et al. 2007). Právě produkce sloučenin s těmito vlastnostmi je charakteristickým rysem pro mnoho druhů rodu *Chromobacterium* (např. Durán and Menck 2001; Han, Han and Segal 2008). Shoda sekvence genu pro 16S sRNA *Chromobacterium* sp. PT79 se všemi kultivovanými i nekultivovanými bakteriemi byla 97 % a nižší. Výskyt tohoto organismu tedy nebyl dosud zaznamenán v žádné jiné ekologické nice. Také *Sporomusaceae bacterium* PT59 (Firmicutes) bylo nalezeno pouze v koloniích mechovky.

Výrazně vyšší diversita bakteriálních druhů byla očekávána v koloniích *P. magnifica*, než v okolní vodě, díky jejímu filtračnímu způsobu přijímání potravy. To ovšem potvrzeno nebylo. Celkově bylo identifikováno 158 izolátů z kolonií mechovky, reprezentujících 43 druhů. Druhů detekovaných ve vodě bylo 42, ale počet identifikovaných izolátů byl méně než poloviční (n = 65). Toto zjištění však není rozhodující ani konečné. Pomineme-li všechna omezení související s kultivační technikou (Článek 1), prostým vysvětlením může být četnost všudypřítomné bakterie *Aeromonas veronii*. *A. veronii* byla nejčastěji detekovaným druhem ve vzorcích vody. Ačkoliv její dominance ve vodě nebyla zvláště výrazná, filtrace pravděpodobně umožňuje kumulaci buněk a zvyšuje převahu *A. veronii* v koloniích. Navýšení celkových počtů kultivovatelných bakterií pak činí izolaci méně početných skupin z nižších ředění nesnadnou, ne-li nemožnou. Kromě toho, jako fakultativní anaerob, je *A. veronii* schopna růstu v aerobních i anaerobních podmínek (Seshadri et al. 2006), aniž by byla potlačena neomycinem o koncentraci 70 mg/l, jak bylo použito v našem experimentu. Skupina *A. veronii*, sdružuje

fylogeneticky velmi podobné organismy, které osidlují širokou škálu obratlovců i bezobratlých hostitelů ve vztahu symbiotickém i patogenním. Rozmanitost prostředí, které *A. veronii* obývá, naznačuje, že je tento druh zobecňován a speciace v důsledku ekologické adaptace může být značná (Silver et al. 2011). Genotypovou rozmanitost populace *A. veronii* ve sledovaných lokalitách Jižních Čech dokazuje variabilita REP-PCR fingerprintových profilů (9.1. Supplementary Figure 2.). V tomto kontextu může být převaha *A. veronii* v koloniích mechovky nejen v důsledku nahromadění, ale také do jisté míry díky adaptaci. Jak již bylo výše zmíněno, kolonie představují ekologickou niku s vyšší úrovní trofizace, což může mít za následek intenzivnější pomnožení těchto bakterií. *A. veronii* je schopná produkovat cytotoxicky (Ghatak et al. 2006) i antimikrobiálně (Odeyemi et al. 2012) působící látky, které by recipročně mohly chránit mechovku před predátory a infekcemi. V rámci naší studie (Článek 4) byla zjištěna antimikrobiální aktivita dvou kmenů *A. veronii* 16/12 a 12/13, které inhibovaly růst lidských fekálních klostridií. *A. veronii* 16/12 také inhibovala růst sympatrického kmene *Pseudomonas moraviensis* 16/12, rovněž izolovaného z *P. magnifica*. Z druhého úhlu pohledu *Aeromonas* spp. jsou častým původcem širokého spektra onemocnění lidí a ryb (Beaz-Hidalgo & Figueras 2013). Masivní expanze *P. magnifica* by mohla představovat zdravotní riziko v rekreačních a hospodářské ztráty v chovných oblastech.

Druhým cílem disertační práce bylo testování antimikrobiální aktivity bakteriálních izolátů (Článek 4) a extraktů připravených z lyofilizovaných kolonií *P. magnifica* (Článek 5). Antimikrobiální aktivita izolátů byla testována agarovou difúzní metodou (Boyanova et al. 2005) proti potenciálně patogenním bakteriím, s ohledem na aplikovaný význam, a proti sympatrickým kmenům, s ohledem na ekologický význam. Jak již bylo výše zmíněno, antimikrobiálně působily dva kmeny *A. veronii* 16/12 a 12/13 inhibující *Clostridium* spp. Lidské fekální klostridie potlačovala také *Pseudomonas moraviensis* 16/12, která byla naopak inhibována *A. veronii* 16/12. V tekutém médiu nebyl po kultivaci aktivních kmenů zaznamenán pokles pH, antimikrobiální aktivita byla tedy pravděpodobně způsobena jinými látkami, než organickými kyselinami. Antimikrobiální aktivita *Aeromonas* spp. proti některým grampozitivním a gramnegativním patogenním bakteriím byla popsána již dříve (Odeyemi et al. 2012), přičemž aktivní kmeny mohou být uplatněny např. jako protektivum proti infekčním onemocněním vodních chovných živočichů (Gibson et al. 1998). *Pseudomonas fluorescens* je producentem antimikrobiálních látek, známých pod anglickým názvem pseudomonic acid A-D. Nejúčinnější z nich je mupirocin (pseudomonic acid A), který působí proti aerobním grampozitivním bakteriím. Méně aktivní je proti gramnegativním bakteriím a anaerobní

grampozitivní bakterie, tedy i klostridie, jsou na mupirocin rezistentní (Sutherland et al. 1985, Class & DeShong 1995). V humánní medicíně se mupirocin osvědčil především při léčbě kožních infekcí a stal se jedním z nejúspěšnějších lokání antibiotik při léčbě *Staphylococcus aureus* v dutině nosní, a to i u kmenů rezistentních na meticilin (Cookson, 1998).

Skutečnost, že antimikrobiální aktivita byla v naší studii prokázána pouze u 3 izolátů, neznamená, že v přirozeném prostředí tomu není jinak. U většiny bakterií sekundární metabolity v *in vitro* podmínkách exprimovány nejsou, přesto, že tato informace je zabudována v genomu. Jejich exprese může být podmíněna celou řadou faktorů, které zahrnují např. složení výživy, působení signálních molekul (elicitorů), selekční tlak, kompetice o místo a živiny, nebo změna redoxního potenciálu (Kleerebezem et al. 1997, Lee et al. 2002, Zigha et al. 2006, Payne et al. 2007, Zhu et al. 2014). V našem experimentu byla testována aktivita bakterií kultivovaných v tekutém médiu, vhodném pro optimální růst daného kmene, a dále variant s přídavkem vodného extraktu z *P. magnifica* a s přídavkem krve. Extrakt z *P. magnifica* ani krev na produkci antimikrobiálních látek vliv neměla. Je třeba zmínit, že extrakt byl sterilován tepelně. Všechny termolabilní bioaktivní komponenty včetně případných proteinových signálních molekul byly inaktivovány, a sledován byl pouze vliv termostabilních nutričních složek.

Z kolonií *P. magnifica* bylo v Ústavu molekulární biologie a farmaceutické biotechnologie Veterinární a farmaceutické university v Brně připraveno 5 různých extraktů: metanolový, hexanový, chloroformový, etyl-acetátový a vodný. Citlivost probiotických, potenciálně patogenních a psychrofilních bakterií způsobujících kažení potravin k výše zmíněným extraktům byla testována agarovou difúzní metodou (Boyanova et al. 2005). U aktivních extraktů byla dále stanovena minimální inhibiční koncentrace mikrodiluční metodou (Wiegand et al. 2008). Antimikrobiální aktivitu vykázaly 3 z testovaných extraktů: metanolový, hexanový a chloroformový. Proti testovaným bakteriím působily v minimálních inhibičních koncentracích (MIC) pohybujících se v rozmezí 0,5 až 10 mg/ml. Citlivé byly především grampozitivní bakterie (*Bacillus cereus*, *Bifidobacterium bifidum*, *Clostridium perfringens*, *Listeria monocytogenes, Micrococcus luteus* a *Staphyloccocus aureus*), přičemž nejvíce citlivá byla patogenní bakterie *Clostridium difficile*, která je zodpovědná za různé formy střevních onemocnění (Abt et al. 2016). Chloroformový extrakt inhiboval také gramnegativní *Pseudomonas aeruginosa*. Antimikrobiální aktivita extraktů z *P. magnifica* byla testována i kolektivem Pejin *et al.* (2015). Na rozdíl od našich výsledků, inhibičně působil proti testovaným bakteriím i etyl-acetátový a vodný extrakt. Pejin *et al.* (2015) také prokázali citlivost *Salmonella*

entirica Typhimurium a *E. coli*, zatímco v naší studii byly tyto bakterie vůči extraktům rezistentní. *B.cereus*, *L. monocytogenes*, *Micrococcus* spp., *P. aeruginosa* a *S. aureus* byly citlivé na extrakty v obou studiích, výrazně se ale lišily hodnoty MIC. Naše hodnoty se pohybovaly v rozmezí 2–10 mg/ml, hodnoty stanovené srbským kolektivem v rozmezí 0,004–0,4 mg/ml. Uvedené rozpětí zahrnuje i hodnoty MIC vodného a etyl-acetátového extraktu, které byly námi shledány jako neúčinné. Testována byla aktivita i další sladkovodní mechovky *Hyalinella punctata* (Pejin et al. 2012). Hexanová frakce z této mechovky, obdobně jako u *P. magnifica*, inhibovala *B. cereus*, *L. monocytogenes* a *S. aureus*. Navíc ale účinkovala také proti *E. coli*, *P. aeruginosa* a *S. entirica* Typhimurium. MIC extraktů z *H. punctata* byly opět výrazně nižší než extraktů *P. magnifica* v naší studii, v některých případech i než ve studii Pejin *et al.* (2015).

Odlišnost v antimikrobiální aktivitě *P. magnifica* z různých geografických lokalit poukazuje na to, že zdrojem aktivních látek není mechovka samotná ani její primární (obligátní) symbionti, jako je tomu u mořských druhů *Bugula neritina* a *Bugula simplex* (Davidson et al. 2001, Lim & Haygood 2004). Primární symbiotické organismy jsou evolučně spjaté se svým hostitelem a díky vertikálnímu přenosu jsou přítomné u všech jedinců bez ohledu na místo výskytu nebo lokální adaptaci (Dale & Moran 2006). Na základě výsledků a dostupné literatury lze dojít k závěru, že producentem sekundárních metabolitů jsou v případě *P. magnifica* především epibiotické či sekundárně symbiotické bakterie, které mechovku kolonizují. Antimikrobiální aktivitu epibiotických bakterií mořských druhů mechovek popsal již Heindl *et al.* (2010). V tomto kontextu je tedy složení bioaktivních látek, spektrum účinku a minimální inhibiční koncentrace buď přímo závislá, nebo do určité míry ovlivněná vnějším prostředím. A to nejen ve smyslu druhového zastoupení mikroorganismů, které mechovku osidlují, ale také mikro- a makroorganismů, které produkci sekundárních metabolitů iniciují či stimulují.

7. Závěr

Kolonie bochnatky jsou bohatým zdrojem dosud nepopsaných bakteriálních taxonů, které jsou snadno kultivovatelné v laboratorních podmínkách. *P. magnifica* je osidlována především alochtonními druhy, běžně se vyskytujícími v prostředí, nebo jsou blízce příbuzné druhům, které jsou méně časté, přesto detekované v ekologických nikách jiných, než jsou mechovci. Díky filtračnímu způsobu příjmu potravy, zachycuje *P. magnifica* bakterie přítomné v okolní vodě a kumuluje je v relativně vysokých počtech. Toho může být využito při izolaci nových druhů bakterií, které běžně není možné ve vodě detekovat, kvůli jejich nízké koncentraci, ačkoliv potenciálně nové taxony byly v rámci studie nalezeny i ve vzorcích vody. Celkem bylo nalezeno 18 bakteriálních fylotypů, které představují 15 potenciálně nových druhů příslušných rodům *Aeromonas*, *Aquitalea*, *Clostridium*, *Herbaspirillum*, *Chromobacterium*, *Chryseobacterium*, *Morganella*, *Paludibacterium*, *Pectobacterium*, *Rahnella*, *Rhodoferax* a *Serratia*, a 3 potenciálně nové rody patřící do čeledí *Clostridiaceae* a *Sporomusaceae*. Z těchto, 2 byly detekovány pouze v koloniích mechovky (*Chromobacterium* sp. PT79 a *Sporomusaceae* bacterium PT 59) a jejich symbiotický vztah nemůže být vyloučen. Stejně tak není možné vyloučit adaptaci, případně fakultativně symbiotický vztah některých dalších druhů, zejména *Herbaspirillum* sp. PT3, *Aquitalea* spp. PT67 a PT99, a *Aeromonas* spp.

Kolonie *P. magnifica* mohou být považovány za slibný zdroj chemických sloučenin, které působí především proti grampozitivním bakteriím. Přesto, že minimální inhibiční koncentrace extraktů byly poměrně vysoké, aktivní extrakty budou dále separovány a jednotlivé frakce opět testovány. Za produkci aktivních látek jsou pravděpodobně zodpovědné fakultativně symbiotické či epibiotické bakterie z prostředí, které mechovku kolonizují, nebo k ní značným způsobem přispívají. Byly nalezeny 2 kmeny *Aeromonas veronii* a 1 kmen *Pseudomonas moraviensis,* u kterých byla antimikrobiální aktivita prokázána.

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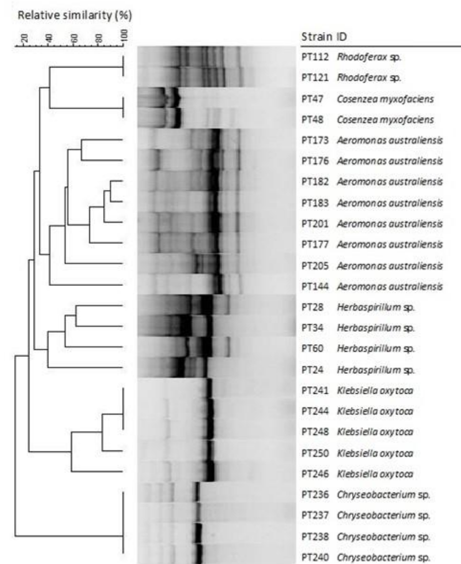
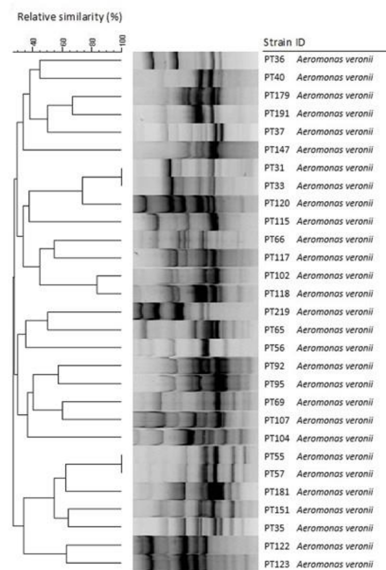
9. Přílohy

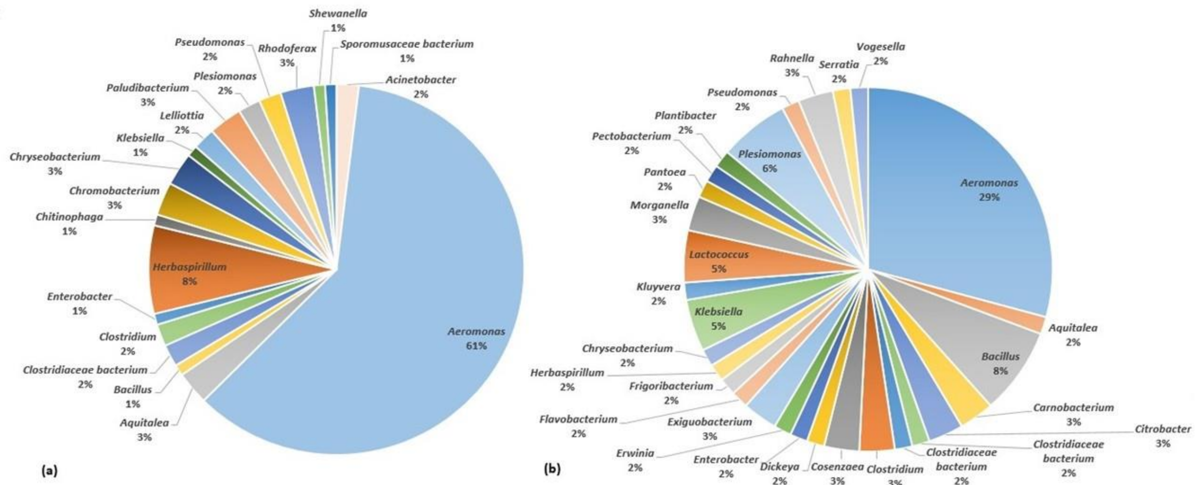
9.1. Doplňující materiály ke článku: Cultivable bacteria from *Pectinatella magnifica* and the surrounding water in South Bohemia indicate potential new Gammaproteobacterial, Betaproteobacterial and Firmicutes taxa.



Supplementary Figure 1. Dendrogram showing the relatedness of selected representative bacterial strains as determined by REP-PCR fingerprint analysis performed using the GTG_5 primer. Relationship between DNA fingerprints were determined by using Pearson's curve-based correlation coefficient and the dendrogram was constructed by using UPGMA clustering method



Supplementary Figure 2. Dendrogram showing the relatedness of *A. veronii* strains as determined by REP-PCR fingerprint analysis performed using the GTG_5 primer. Relationship between DNA fingerprints were determined by using Pearson's curve-based correlation coefficient and the dendrogram was constructed by using UPGMA clustering method



Supplementary Figure 3. Distribution of bacterial genera isolated from Pectinatella magnifica (a) and surrounding water (b). NOTE Haemophilus piscium (syn Aeromonas salmonicida) is counted in the genus Aeromonas.