

## Transbiome Invasions of Femtoplankton

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Received June 30, 2015; in final form, September 17, 2015

**Abstract**—The results of ecological and phylogenetic studies of femtoplankton, the smallest size fraction of plankton formed by viruses and ultramicrobacteria (UMB), are overviewed to shed light on the problem of transbiome invasions by microbes. Phylogenetic lineages of viruses and UMB are shown to be associated with particular biomes, thus indicating infrequent transbiome transitions in the microbial world. An alternative hypothesis of widespread cross-colonization events requires a deeper analysis of the factors that form the barrier between biomes and are responsible for the adaptation of microorganisms to different environments.

**Keywords:** femtoplankton, virioplankton, ultramicrobacteria, filterable bacteria, SAR11, LD12, phylogenetic diversity, biome, salinity, osmoregulation

**DOI:** 10.1134/S1995425516030112

**Femtoplankton** is the smallest (less than 0.2  $\mu\text{m}$ ) and least studied size fraction of plankton comprising viruses and filterable bacteria (so-called *ultramicrobacteria*). The ability of these forms to pass through the filters traditionally used to separate the particulate organic matter (POM) and dissolved organic matter (DOM) formally allows them to be regarded as DOM. As it has emerged, the “live DOM” is among the key players in the control of global biogeochemical cycles. A better understanding of femtoplankton significance in the function of aquatic ecosystems has been associated with the developed concept of ‘microbial loop’ and, later, ‘viral loop’ (Nagata and Kirchman, 1992), sometimes referred to as the viral “shunt,” since this term better reflects the essence of the virus control of the nutrient and energy fluxes in aquatic ecosystems.

Only recently viruses have been regarded as an obligatory element in the structure of an aquatic ecosystem and its material flows. According to the modern concept, their primary ecological function consists of the redistribution of material flows from the microbial loop to the DOM and POM pools as a result of the lysis of plankton organisms that they induce. Thus, the virus shunt “works” against the microbial loop leading to a “short circuit” in the structure of matter and energy flows. The virus-controlled matter recirculation is accompanied by considerable energy losses (thermal dissipation), since the host metabolism provides the production of new viruses in a water column (thereby also supplementing the DOM pool). This process, seemingly adverse for aquatic ecosystems, has emerged to be exclusively favorable for the maintenance of biodiversity and its increase. Viruses

can intensify the metabolism, immunity, and adaptive capabilities of their hosts; influence their distribution and evolution (Rohver and Thurber, 2009); and control their species diversity (Martiny et al., 2014).

The genetic diversity of viruses themselves is impressive: metagenomic analysis demonstrates that 200 L of water can simultaneously contain 3000–7000 virus genomes (Breitbart et al., 2004). Although intensive studies into this phenomenon are commenced only recently, the key to understanding lies in a comparison of the genetic diversity and specific ecological features of the virioplankton from different biomes, namely, soils as well as marine and freshwater bodies.

**Viruses.** Armed with state-of-the-art tools for the detection and quantitative assessment of virus abundance, researchers over a short time period have accumulated a considerable volume of the data on virus communities in different biomes and habitats, including oceanic depths, solar salterns with the water salinity exceeding the marine level several tenfold, acidic hot springs, alkaline (pH 10) polar lakes, and groundwaters to a depth exceeding 2000 m (Anderson et al., 2013). A preliminary estimation of the published data, including phylogenetic analysis, suggests the following inferences, some of which are contradictory: (1) the virus abundance in some water bodies is likely independent of salinity but is associated with quantitative characteristics of primary and secondary producer development (that is, water trophy), as well as seasonal cycles (Wommack and Colwell, 2000); (2) the role of freshwater virioplankton in the regulation of carbon and nutrient fluxes, as well as the structure of micro-

bial communities, has been studied to a considerably lesser degree, but the available data are sufficient to state that the general postulates in the concept of viral shunt elaborated for marine systems are similarly true for freshwater bodies; (3) the microbial mortality caused by virus infection and consumers has been better studied in marine ecosystems; (4) the genetic diversity of marine viruses has been described in considerably more detail than that of freshwater viruses (Short and Suttle, 2005); (5) the accumulated volume of phylogenetic information about the aquatic viruses suggests that the viruses of closely related microorganisms from the freshwater and marine biomes are genetically separated (Wilhelm and Mattison, 2008); (6) closely related host microorganisms and the phages infecting them are widespread in different biomes, and the horizontal gene transfer takes place between phage communities from different biomes (Short and Suttle, 2005); and (7) marine and freshwater microorganisms (and viruses) are typically rather distant from a genetic standpoint and frequently group into separate marine and freshwater clusters analogously to macroorganisms.

The listed general statements are contradictory and leave considerable ambiguity and many questions, first and foremost, about the factors that provide the barriers between the marine and freshwater biomes, as well as enhance the environment-specific divergence and adaptation. A comparative analysis of the communities from the biotopes of the hypersaline–marine–brackish–freshwater gradient could be most promising for solving this research problem.

**Ultramicrobacteria (UMB).** The prokaryotic component of femtoplankton, mainly represented by UMB, was for a long time *terra incognita* because of methodological hindrances in its study and an underestimation of its significance in biogeochemical cycles of seas and freshwater bodies (the total biomass of community is small). The research in this area have been intensified only recently thanks to the application of molecular methods to unculturable bacteria. The specific features in UMB metabolism and the associated pattern of UMB distribution in biotopes have been partially clarified (Williams et al., 2011); however, the issues associated with UMB involvement in the microbial loop and their susceptibility to virus infections (that is, their role in the viral shunt) are still disputable. Several studies have shown that the minute cell size allows UMB to escape being consumed by phagotrophic protozoans (Yooseph et al., 2010), which may form the basis for the hypothesis on the exclusion of UMB from the microbial loop. Another hypothesis arises; according to it, the prokaryotic femtoplankton is resistant to virus infection, since putatively dormant cells with a low metabolism constitute most of it (Gazol et al., 1995). Both abovementioned UMB defense mechanisms may lead to a considerable decrease in the UMB mortality rate, that is, to the formation of a certain “survival depot” of bacterioplankton. The hypothesis on existence of such a depot is still

to be verified; however, the experimental data refuting it, namely, that at least part of UMB community are physiologically active cells, have been already obtained (Mukhanov and Kemp, 2005; Mukhanov et al., 2007).

The mystery of the nature of these most minute prokaryotes is that the volumes of their cells correspond to or are even smaller (as in the so-called *nanobacteria*) than the hypothetical limit that can house the minimal set of organelles and other components necessary for its normal function and reproduction (Velimirov, 2001). The existence of *nanobacteria* able to calcify, first discovered in geological specimens (Folk, 1999) and then identified as dangerous human pathogens (Kajander and Ciftcioglu, 1998), is subject to considerable doubts (Urbano, P. and Urbano, F. 2007); however, this does not interfere with the boom of clinical microbiological studies of these forms during the last decade (Alenazy et al., 2014; etc.). If nanobacteria are actually prokaryotic (or other) cells, they should be necessarily present in aquatic medium similar to any microbial pathogens entering coastal waters with utility discharge. In this case, *nanobacteria* should be a constituent of femtoplankton along with dormant pathogens. This problem has still not been set in any research. Since the research into this and other most minute forms (for example, *ultramicroarchaea*, *nanoarchaea*, *nanobes*, etc.) is, as a rule, separately conducted by specialists in different areas of general and clinical microbiology, it is rather a problem to use particular terminology discussing the prokaryotic femtoplankton (Duda et al., 2012).

Although the term ultramicrobacteria is rather universal, it is typically used to describe the *autochthonic* bacteria ubiquitous in all aquatic ecosystems with extremely small cell volume ( $<0.1 \mu\text{m}^3$ ) and genome (3.2–0.58 Mb) belonging to Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes. In particular, these filterable forms include numerous dormant bacteria able to restore their active growth and increase their cell volume with the advent of favorable conditions (referred to as ultramicrocells by some researchers).

According to a narrower definition of UMB, which is becoming prevalent in aquatic microbiology in the last decade, these bacteria prefer oligotrophic water with a low content of nutrients and organic substances. At a small total biomass (and comparatively high population), the reproduction of these prokaryotes requires a smaller amount of nutrients. In addition, the UMB mortality rate can be lower than that for “traditional” bacterioplankton.

Similar to any minute bacteria, the marine UMB make up a considerable part of the SAR11 clade (or Pelagibacteraceae). They prefer oligotrophic conditions and are widespread in seas and oceans; they account for 25–50% of all prokaryotes (Morris et al., 2002). The SAR11 bacteria are identified as alphaproteobacteria and include *Pelagibacter ubique* (Rappé et al., 2002),

one of the most abundant marine bacterial species. They use dissolved organic carbon and nitrogen as a substrate (Morris et al., 2002) and are unable to fix carbon or nitrogen, but synthesize all amino acids except for glycine (Tripp et al., 2009) and some cofactors (Giovannoni et al., 2005). One of the riddles in SAR11 metabolism is their demand in reduced sulfur (Tripp et al., 2008). In addition, *P. ubique* contains protorhodopsin, necessary for ATP synthesis, although these bacteria are not phototrophs.

Alphaproteobacteria is a common component of the marine bacterioplankton community, which was earlier assumed to be infrequent in lakes. Nonetheless, the unculturable freshwater alphaproteobacterium LD12 strain has emerged to form a monophyletic group closely related to SAR11 bacteria. Molecular genetic analysis has demonstrated their high abundance and ubiquitousness in the freshwater biome (Salcher et al., 2011). The LD12 bacteria are likely to prefer oligotrophic conditions with low nutrient concentrations; however, their number in the epilimnion during the summer season can exceed  $5 \times 10^8$  cells  $L^{-1}$ , which is comparable to the UMB abundance in seas (Salcher et al., 2011). In their phenotypic characteristics and specific metabolic features, LD12 bacteria are similar to *Pelagibacter* (Rappé et al., 2002) and presumably occupy similar ecological niches in freshwater bodies. These groups together form one of a few UMB monophyletic lineages that had succeeded in passing the barrier between marine and freshwater habitats.

Several papers postulate that this most pronounced prevalence of the SAR11 bacteria (or pelagiobacteria) in the marine ecosystems results from an unusual SAR11 UMB resistance to phage infection because of their minute cell size, the so-called cryptic escape (Yooseph et al., 2010), and/or the defense specialization of K strategists (Suttle, 2007). The alternative hypothesis is that an increase in the cell specific surface with their diminishing and a decrease in the size of their genome during the evolution gave rise to unusually efficient metabolic processes providing DOM oxidation. In turn, this allowed the minutest eukaryotes to maintain relatively high abundance even if they are susceptible to bacteriophages. This hypothesis was recently discussed in one of the *Nature* publications (Zhao et al., 2013). The authors succeeded in obtaining some indirect evidence by discovering highly abundant SAR11 viruses in sea water, which they correspondingly named pelagiophages. As has emerged, the pelagiophage genomes are widely represented among the marine virus metagenomes, suggesting their significance in the marine ecosystems. One of the discovered phages, HTVC010P, is present in all datasets and is thus affiliated with the new podovirus subfamily, which can well be regarded as the most numerous virus subfamily in the biosphere. This discovery refutes the hypothesis that SAR11 bacteria (and UMB) are immune to phages and, correspondingly, confirms the idea that these forms have compet-

itive advantages in the struggle for resources. Nonetheless, the question still remains open, because the UMB physiology and bioenergetics (especially after infection) are vague. Since the largest part of the UMB in the plankton femtofraction is likely to consist of dormant cells with a low specific rate of metabolic processes, their successful infection by phages is rather doubtful. The situation with the freshwater LD12 UMB is also unclear as for whether discovery of specific LD12 phages can be expected. The answers to these questions are to be found in the future.

**Are microorganisms able to easily pass the transbiome barriers?** Unlike the majority of macroorganisms, the sizes of microbial populations are considerably larger; moreover, they can be transferred to large distances. Thus the microbial populations have a tremendous potential for passive spreading (Falkowski et al., 2008). The reproduction rate of their populations is incomparably higher (Weisse, 2008). Finally, their stunning genetic diversity (Rusch et al., 2007) considerably elevates their adaptation potential. All these properties might be thought to suggest the possibility and ability of microorganisms to easily cross the biome boundaries and colonize new biotopes. Indeed, there is a belief that sea and freshwaters were colonized by the same microbial taxa (for review, see (Hahn, 2006)). However, this assumption still remains a hypothesis requiring verification.

The boundary between the marine and freshwater biomes, of which a jump in salinity is characteristic, is one of the most difficult-to-overcome barriers. The osmotic pressure gradients and ion concentrations are the most important factors that limit the cross-colonization of marine and freshwater biomes by animals and plants. Colonization of a new biome, first and foremost, implies a considerable increase in the organism's expenditures for osmoregulation (Oren, 2001). However, the presence of competitors and predators in the colonized biome can also be of great importance, along with the differences in salinity (Vermeij and Dudley, 2000).

When colonizing a habitat with new conditions, microorganisms encounter the same physiological or energy problems. The presence of a well fit aboriginal community can also considerably interfere with microbial colonization (De Meester et al., 2002). Nonetheless, it is still unclear how easily the microorganisms can pass the salinity barrier.

Numerous phylogenetic studies of manifold microbial groups (bacteria, archaea, microeukaryotes, and viruses) suggest that the boundary between marine and freshwater biomes presents a serious barrier to them. Before the advent of molecular methods, the freshwater and marine microorganisms were believed to insignificantly differ at least in their taxonomy and functional characteristics; however, the new data of phylogenetic analysis involving 16S rRNA (for the prokaryotes) and 18S rRNA (for microeukaryotes) has changed this view.

It becomes clear that the marine and freshwater biomes are populated with separate prokaryotic communities. Although there are rather few data on archaea, the difference between their marine and freshwater strains was long beyond doubt. Later, these assumptions were confirmed by field studies of the archaeal communities from rivers, lakes, and Arctic coastal waters (Galand et al., 2008). Nonetheless, some Archaea phylogenetic lineages, for example, Crenarchaeota, were able to easily cross the biome boundaries (Galand et al., 2008).

The ever increasing number of phylogenetic studies suggests the existence of well-distinguishable phylogenetic lineages of freshwater bacteria in the overall range of the known taxa (for review, see (Hahn, 2006)). Bacterial strains of different geographical origins, sometimes from very distant habitats, fall into the same cluster uniting the microflora of one biome. This pattern was for the first time demonstrated for SAR11 ( $\alpha$ -proteobacteria), one of the microbial groups widespread in the marine biome. Within SAR11, the sequences of freshwater species cluster together, forming the LD12 subcluster (Zwart et al., 2002; Kan et al., 2008). The first LD12 representatives were discovered in Toolik Lake, Alaska, and then have been found in freshwater all over the world (Zwart et al., 2002), possibly suggesting that they have colonized the freshwater biome and subsequently evolutionarily diverged as a result of geographical expansion. Recent phylogenetic studies have also confirmed the existence of a SAR11 cluster associated with brackish waters (Kan et al., 2008). This SAR11 “structuredness” is amazing, since these microorganisms form the basis of surface oceanic bacterioplankton and their abundance in the World Ocean is tremendous, being estimated as  $2.4 \times 10^{28}$  cells (Morris et al., 2002).

The separation of marine and freshwater taxa has been also demonstrated for other bacterial groups. In particular, 34 phylogenetic clusters have been distinguished for the freshwater organisms (Zwart et al., 2002); these clusters contain  $\alpha$ -,  $\beta$ -, and  $\gamma$ -Proteobacteria; Bacteroidetes; Cyanobacteria; Actinobacteria; Verrucomicrobia; green nonsulfur bacteria; and unculturable OP10 bacteria. The term “typical freshwater bacteria” was introduced; actually, this term implies a low probability of transbiome bacterial transitions.

Analogous phylogenetic comparison of the viruses from different biomes is hindered by an imbalance in the volumes of accumulated data, since the freshwater viruses are very poorly studied. However, new results suggest a considerable genetic distinction between the virus communities inhabiting sea and freshwaters. In particular, several studies of cyanophage genes *g20* and *psbA*, encoding a structural protein and the D1 protein, involved in oxygenic photosynthesis, respectively, demonstrate that these genes strictly cluster with either freshwater or marine groups (Short and Suttle, 2005). A similar pattern has been observed for

the podoviruses (similar to T7 bacteriophages) (Breitbart et al., 2004) and picodnaviruses infecting unicellular eukaryotic phytoplankton (Short, S.M. and Short, C.M., 2008). Thus, a pronounced association of individual phylogenetic lineages to a particular biome is also evident in the case study of viruses; in turn, this suggests that the transbiome transitions are a rare phenomenon. This is quite expected taking into account the fact that the viruses are completely dependent on their hosts and their habitat preferences. However, other factors can play an important role, since virus immigration to a biome has a tremendous effect on the structure of virus communities (Snyder et al., 2007). Freshwater viruses can successfully replicate in marine systems (Sano et al., 2004). This means that at least some of them may have a wide range of the hosts abundant in different biomes.

Despite the evidence-based background and conceptual integrity, the idea about the isolation of microbiota of different biomes has its opponents. In particular, Breitbart and Rohwer (2005) expressed an alternative point of view in their review titled “Here a Virus, There a Virus, Everywhere the Same Virus?” The authors assume that the local diversity of a microbial community in any place would be rather high and the global diversity rather low under conditions of transbiome virus “migration.” Vice versa, if the biomes are isolated, each local biotope would contain unique endemic virus populations and the global virus genome would be estimated as 100 million unique genotypes based on metagenomics data (Rohwer, 2003). As the authors see it, the correct hypothesis can be chosen, for example, by finding out whether identical or almost identical sequences of conserved bacteriophage genes are present in different biomes (Short and Suttle, 2005; Breitbart et al., 2004). One of the phage DNA polymerase sequences, named HECTOR, was found in marine water, cattle rumen fluid, the surface of corals, and water of solar salterns (Breitbart et al., 2004), which may suggest that it relatively recently (on an evolutionary scale) crossed the biome boundaries. Assuming that the average phage yield and the half-life of virus particles are 25 particles and 48 h, respectively (Wommack and Colwell, 2000), the reproduction of the population of a phage carrying the HECTOR sequence would require one replication per 10 days (i.e., over five half-life periods) with the annual yield of 36 generations. The phage mutation rate is approximately  $10^{-7}$  to  $10^{-8}$  per DNA base pair per one generation (Sniegowski et al., 2000). Consequently, one change in a base pair of the HECTOR sequence is expected approximately every 525 years. Since the sequence of 533 bp has been never changed by more than three pairs, we can state that HECTOR crossed the biome(s) boundary over the last 1000–2000 years.

It is unknown whether the overall HECTOR-carrying phage is transferred or only the corresponding DNA fragment. At least, it is known that viruses are

able to find hosts in biomes foreign for them (Sano et al., 2004), which can be explained by the presence of the same host microorganisms in different biomes and/or the ability of viruses to attack a wide range of hosts in each biome (Sullivan et al., 2003).

**The prospects of studying transbiome invasion by femtoplankton.** Although the hypothesis stating that the biomes are isolated and that the barriers between the marine and freshwater biomes are hardly passable by microorganisms looks better evidenced, the corresponding question is still open and requires further studies. First and foremost, it is necessary to clarify the factors that form this barrier between the biomes and are responsible for microbial divergence and adaptation to new conditions. Comparative studies of the microbial communities in seas and salt lakes could allow salinity to be excluded from the factors that form the barrier between biomes to focus on the barriers of ecological nature. Salinized lakes, having evolved from freshwater to salt state, are also promising model systems for comparing the genetic diversity of their microflora at different stages of salinization, as well as of the microbial communities of adjacent freshwater lakes and sea water. Such studies would give new information about the physicochemical and ecological mechanisms underlying the establishment of the barrier between marine and freshwater biomes, the more so since hypersaline and desalinated water bodies are still beyond the focus of molecular microbiologists.

Another similarly promising area in studying the phenomenon of transbiome (marine–freshwater) invasion of microorganisms could be an analysis of the genes responsible for osmoregulation in closely related strains from different biomes. Phylogenetic studies based on the information about these genes would provide more accurate estimates for the number and duration of transbiome transition events for individual taxa, as well as a better understanding of the role of salinity in genetic divergence and adaptations of microorganisms.

#### ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, projects nos. 14-04-90421 and 14-44-04158 r\_sibir\_a.

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Translated by G. Chirikova