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Abbreviations:

	EPA	Eicosapentaenoic acid (20:5 ω-3)
	DHA	Docosahexaenoic acid (22:6 ω-3)
	AA	Arachidonic acid (20:4 ω-6)
	ω-3	Omega-3
	PUFA	Polyunsaturated fatty acid

ABSTRACT

Trichomonas vaginalis is a sexually transmitted parasite and, while it is often asymptomatic in males, the parasite is associated with disease in both sexes. Metronidazole is an effective treatment for trichomoniasis, but resistant strains have evolved and, thus, it has become necessary to investigate other possible therapies. In this study, we examined the effects of native and oxidized forms of the sodium salts of eicosapentaenoic, docosahexaenoic and arachidonic acids on *T. vaginalis* activity. Eicosapentaenoic acid was the most toxic with 190 μM and 380 μM causing approximately 90% cell death in Casu2 and ATCC 50142 strains, respectively. In contrast, oxidized eicosapentaenoic acid was the least toxic, requiring >3 mM to inhibit activity, while low levels (10 μM) were associated with increased parasite density. Mass spectrometric analysis of oxidized eicosapentaenoic acid revealed C20 products containing one to six additional oxygen atoms and various degrees of bond saturation. These results indicate that eicosapentaenoic acid has different effects on *T. vaginalis* survival, depending on whether it is present in the native or oxidized form. A better understanding of lipid metabolism in *T. vaginalis* may facilitate the design of synthetic fatty acids that are effective for the treatment of metronidazole-resistant *T. vaginalis*.

Keywords
Disease; Fatty Acid/Oxidation; Fish Oil; Infection; Lipids; Omega-3 Fatty Acids; Polyunsaturated Fatty Acids; Trichomoniasis

INTRODUCTION

Trichomonas vaginalis was first identified by Donné in 1836, and is a protozoan microaerophilic parasite that causes human trichomoniasis (Petrin et al. 1998). Trichomoniasis is the most common non-viral sexually transmitted infection, as illustrated by the fact that there are approximately 180 million cases per year worldwide, with about 8 million new cases occurring in the U.S. every year (Schwebke 2005). The disease affects both women and men, but is slightly more prevalent in women than men, and is disturbingly high in certain racial or ethnic groups (Miller et al. 2005). *T. vaginalis* thrives in the lower genital tract of both males and females and, notably, 70–85% of those infected are asymptomatic. It is considered to be a "neglected" parasitic infection, because it is often left untreated, leading to an increased risk of many other health problems (Cotch et al. 1997; Saurina and McCormack 1997; Schwebke and Burgess 2004; Stark et al. 2009; Thurman and Doncel 2011; Meites 2013). For women, there is an increased prevalence of other sexually transmitted infections, adverse outcomes of pregnancy, and pelvic inflammatory disease (Cotch et al. 1997; Moodley et al. 2002; Allsworth et al. 2009). In addition, *T vaginalis* pseudocysts have been isolated from patients with cervical cancer, indicating that the parasite may play a role in the pathogenesis of this disease (Afzan and Suresh 2012). Trichomoniasis in men may be underestimated, since the methods used for its detection are not very sensitive (Kaydos-Daniels et al. 2004). In men, *T. vaginalis* is found to colonize the prostate and is correlated with an increased risk of aggressive prostate cancer (Stark et al. 2009; Twu et al. 2014). Furthermore, men with trichomoniasis, who are also infected with HIV, displayed a higher concentration of HIV RNA in their seminal fluid, and thus pose an increased risk of transmission of HIV (Hobbs et al. 1999).

The only recommended regimens approved by the FDA for the treatment of trichomoniasis involve the use of 5-nitroimidazole antimicrobials (Meites 2013). Treatment consists of either metronidazole (marketed as Flagyl; 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol) or tinidazole (marketed as Tindamax, Fasigyn and Simplotan; 1-(2-ethylsulfonylethyl)-2-methyl-5-nitro-imidazole) (Workowski and Berman 2010). Use of metronidazole has a number of side effects, including both peripheral and central nervous system-associated neurotoxicity, although these effects appear to be reversible (Sarna et al. 2009). Furthermore, metronidazole is teratogenic and its use in pregnancy has been controversial (Nanda et al. 2006). Reports of metronidazole-resistant strains, however, have been on the rise since first reported in 1962 (Robinson 1962; Krajden et al. 1986; Cudmore et al. 2004). Although effective in some treatments, tinidazole is not effective against all metronidazole-resistant isolates (Narcisi and Secor 1996). Thus, the treatment of trichomoniasis presents a challenging clinical problem.

To date, very little is known concerning the effect of lipid mediators on parasites such as *T. vaginalis*. Furthermore, there are no reports concerning the effect of eicosapentaenoic acid (EPA, C20:5ω-3) on this parasite. EPA is an omega-3 polyunsaturated fatty acid (PUFA) that is found in fish oil and which cannot be synthesized *de novo* by humans. Increased intake of fish oil has been found to increase the host's ability to combat inflammation, infection and disease (Connor and Connor 1997; Alexander 1998; Kris-Etherton et al. 2002; Leaf et al. 2003; Barnham et al. 2004; Lukiw et al. 2005; Wong 2005; Calder 2006; Yokoyama et al. 2007). Interestingly, PUFAs such as EPA, arachidonic acid (AA) and docosahexaenoic acid (DHA) are reported to

have anti-parasitic activity, causing the death of *P. falciparum* in vitro (Kumaratilake et al. 1992; Arun Kumar and Das 1999). Furthermore, there is some evidence that the host-parasite interaction can be modulated by dietary PUFA (Taylor et al. 1997; Schlotz et al. 2013). The fatty acids may act directly on the parasites, although EPA may also activate neutrophils and macrophages in the host resulting in increased killing of the parasites (Kumaratilake et al. 1997).

In this study, we examined the effects of the sodium salt of EPA, DHA and AA in the native and oxidized forms on *T. vaginalis* survival. Our results indicate that *T. vaginalis* responds differently to native and oxygenated PUFAs, and that fatty acids that incorporate the optimum structure requirement may hold promise as future alternative targets for the treatment of metronidazole-resistant *T. vaginalis*.

MATERIALS AND METHODS

Materials

Cis-5,8,11,14,17-eicosapentaenoic acid sodium salt (> 99+%), *cis*-4,7,10,13,16,19-docosahexaenoic acid sodium salt (≥ 95%), and *cis*-5,8,11,14-eicosatetraenoic acid sodium salt (arachidonic acid sodium salt; ≥ 99%) were purchased from Sigma-Aldrich (Missouri, USA) and stored in the solid form as received under an inert atmosphere at -80 °C. Henceforth, "EPA", "DHA" and "AA" denote the sodium salts of EPA, DHA and AA, respectively.

Culture of *T. vaginalis*

In this study, we used *T. vaginalis* isolates that are metronidazole sensitive (Casu2, also designated as SS-22; isolated 2008, Sardinia, Italy) (Strese et al. 2014) and metronidazole resistant (ATCC 50142, also designated as RU 393, isolated 1983, New York, U.S.) (Muller et al. 1988). *T. vaginalis* parasites were maintained in tryptose/yeast extract/maltose (TYM) medium, pH 6.2 (Diamond 1957) containing 10% (v/v) heat-inactivated horse serum, at 37 °C. The stock cultures and experimental samples (5.5 mL) were maintained in screw-top glass tubes (16 x 125 mm, 16 mL capacity).

Growth of *T. vaginalis* under ambient aerobic and anaerobic conditions

T. vaginalis microbes were grown under ambient aerobic conditions at 37 °C to levels ≥ 1 x 10^6 mL^{-1} in TYM media containing 10% horse serum (Lehker and Alderete 1990). *T. vaginalis* samples (ATCC 50142) were incubated with or without native EPA (10 μM and 100 μM) in TYM medium containing horse serum (2% v/v). The experiments were initiated with approximately 2 x 10^4 parasites/mL. The lower levels of serum and parasite density facilitated our ability to observe the direct effect of EPA on parasite activity and to monitor parasite viability, respectively. In addition, the reduced amount of serum allowed us to better assess the direct effect of EPA on the parasite, without any interference from the serum. For experiments under ambient aerobic conditions, samples were prepared in air with no attempt to exclude oxygen, and placed in an incubator at 37 °C for up to 48 h. These samples are designated as "aerobic" samples. The concentration of oxygen in the air-saturated medium at 37 °C is reported as 210 μM, and is expected to decrease over time, as it becomes metabolized by the parasites (Paget and Lloyd 1990). For experiments under anaerobic conditions, samples were placed in an

Oxoid Anaerojar (2.5 L capacity; Thermo Scientific™, North Carolina, USA) in the presence of a GasPak™ EZ Gas Generating Sachet (Becton, Dickinson and Company, Maryland USA) that allows the creation of an anaerobic atmosphere. The Oxoid Anaerojar was placed in an incubator at 37 °C up to 48 h. Samples were prepared in triplicate and experiments were replicated at least three times.

Parasite counts

Viable parasites were counted by placing resuspended culture media (20 μL) on an improved Neubauer hemocytometer (1/400 sq. mm.) at various time points. Only viable parasites were included in the counts. Counts were performed in triplicate and averaged.

In vitro minimum inhibitory concentration (MIC) assay

The effect of different concentrations of native EPA, DHA and AA and their oxidized forms (oxEPA, oxDHA and oxAA) on the activity of *T. vaginalis* isolates (ATCC 50142 and Casu2) was determined using an *in vitro* minimum inhibitor concentration (MIC) assay performed in a 96 well plate. TYM supplemented with 10% (v/v) horse serum was introduced to each well of the plate (50 μL per well). Next, native PUFA or oxPUFA (50 μL; approximately 8 mg/mL in molecular grade water) was added to the first well of three rows. PUFA or oxPUFA were serially diluted along the same row of the plate in the following manner: 50 μL of the solution in well 1 containing the test compound was transferred to the second well of the same row, and the process repeated for wells 3 through 12. The final 50 μL was discarded. *T. vaginalis* cultures were diluted to approximately 5 x 10^5 cells/mL in TYM containing 10% horse serum and 150 μL of this solution was added to each well, such that the total volume of each well was constant at 200 μL. The test compound concentrations examined included: 12 μM, 24 μM, 48 μM, 96 μM, 190 μM, 380 μM, 760 μM, 1.5 mM, 3.0 mM, and 6.0 mM. These concentrations represent a range of solutions containing 4 μg/mL–2 mg/mL PUFA or oxPUFA. Two separate rows served as control containing vehicle. The plates were incubated at 37 °C and examined microscopically at 24 h.

Growth curves

T. vaginalis strains ATCC 50142 and Casu2 were cultured in TYM media supplemented with 10% (v/v) horse serum in glass screw-cap tubes as described above. For the determination of growth curves, tubes were combined and diluted with TYM media containing 10% (v/v) horse serum to give an initial cell count of 4 x 10^5 parasites/mL for each strain. The ATCC 50142 and Casu2 cultures were each split into two volumes for each strain, with one volume serving as control and the other incubated with oxEPA (10 μM). The parasites from the four groups (*i.e.* ATCC 50142 and Casu2 strains with and without oxEPA) were aliquoted into several sterile glass screw-cap tubes (5 mL/tube) and placed into a 37 °C incubator. For each time point, a separate tube was removed from the incubator for the relevant group and viable parasites counted as described above.

Mass Spectrometry

Native EPA was prepared immediately before use by dissolving solid EPA sodium salt (0.5 mg) in molecular grade water (1 mL; distilled; deionized; DNAse, RNAse, and protease tested) from Cellgro® Mediatech, Inc. (Vermont, USA), purged with nitrogen gas. Oxidized EPA (oxEPA) was prepared by exposing solid EPA sodium salt (0.5 mg) to air at ambient temperature (22.2–

25.4 °C) for three days before dissolving the solid in molecular grade water to form solutions (0.17 mM EPA or oxEPA) that were filtered (0.22 μm Millex-GP filter unit; EMD Millipore Corporation; Maryland, USA). Native EPA and oxEPA samples were analyzed using an Applied Biosystems MDS SCIEX API 2000 instrument. The mass spectrometer was operated in negative ion mode with a mass range of m/z 250 to 650 amu, using a declustering potential (DP) of -60.0V, a focusing potential (FP) of -400.0V and an entrance potential (EP) of -100.0V. Nitrogen was used as both the sheath gas and the auxiliary gas. Data acquisition and analysis were performed using Analyst software, version 1.4. Samples were introduced *via* syringe injection at a flow rate of 10.00 μL/min. Spectra (100 cycles) were accumulated over a 5 min period.

Statistical analysis

Results are presented as means (± standard error of the mean, SEM) with significant differences determined by a one-way analysis of variance (ANOVA) test. In cases where we obtained a significant one-way ANOVA result, Tukey's Multiple Comparison test was applied (as a post-hoc test) to determine where the significant differences occurred between the groups. All statistical analyses were performed using GraphPad Prism 4.0a software (La Jolla CA, USA).

RESULTS

The toxic effects of native EPA on T. vaginalis survival under both ambient oxygen and anaerobic conditions

Parasite motility counts demonstrate the effect of native EPA on *T. vaginalis* (strain ATCC 50142) growth at 24 and 48 h (**Fig. 1a**). Under ambient oxygen conditions, EPA (10 and 100 μM) caused significant decreases in *T. vaginalis* populations at 24 and 48 h compared to control. Indeed, the higher concentration of EPA (100 μM) caused complete inhibition of parasite growth at 48 h. A change in morphology from pear-shaped trophozoites to those classically described as pseudocysts with a more rounded shape were seen after incubation with native EPA. These pseudocysts, which are living cells but without apparent motility, have been reported to appear under stressful environmental conditions (Pereira-Neves et al. 2003) and were followed by death of the parasite within a few hours.

To explore the relationship between the effect of EPA on the parasite and the presence of oxygen, we incubated *T. vaginalis* (strain ATCC 50142) with native EPA in the absence of oxygen (**Fig. 1b**). Under anaerobic conditions, significant decreases in *T. vaginalis* populations were observed at 24 and 48 h compared to control, with complete death of the parasite occurring at 48 h under higher EPA concentrations (100 μM). Although EPA proved detrimental to the survival of the parasite irrespective of oxygen concentration, a ratio of counts of parasites under aerobic versus anaerobic conditions reveals differences in the rates of growth of samples (**Fig. 1c**). Under aerobic conditions, maximal growth was observed at 24 h, whereas under anaerobic conditions, parasite growth was greater at 48 h. Trace levels of oxygen (< 0.25 μM) enhance parasitic growth, but our ambient oxygen concentrations in solution are much higher (210 μM at 37 °C) (Paget and Lloyd 1990). Our data indicate a more rapid growth and subsequent faster decline in parasite numbers under aerobic versus anaerobic conditions, suggesting a greater sensitivity to these oxygen levels (**Fig. 1**). However, despite these differences in growth rates,

native EPA was toxic to the parasite regardless of the presence of oxygen. The differences in growth rates may be the result of pH changes. Under aerobic conditions, the parasite produces lactate and acetate, which will lower the pH but, under anaerobic conditions, the parasite produces glycerol and lactate, which will take longer to decrease the pH and, hence, presumably account for the continuous increase in parasite numbers out to 48 h.

The anti-parasitic activity of PUFAs and oxidized PUFAs

To determine whether other lipids exert a similar effect to EPA, we investigated the anti-parasitic activity of docosahexaenoic acid (DHA; 22:6 ω-3), and arachidonic acid (AA; 20:4 ω-6), along with EPA in an MIC assay. The effect of a range of native EPA, DHA and AA concentrations on the activity of *T. vaginalis* isolates ATCC 50142 and Casu2 was tested and the results demonstrate that of the three PUFAs, EPA was the most effective at killing *T. vaginalis* (**Fig. 2**). In all cases, lower concentrations of PUFAs were required for killing the Casu2 versus the ATCC 50142 isolates. At 24 h, approximately 90% of the Casu2 parasites were non-viable using 190 μM EPA (**Fig. 2a**), whereas for the ATCC 50142 isolates, slightly higher levels of 380 μM EPA were required (**Fig. 2d**). At 48 h, 90% cell death was achieved with 48 μM and 96 μM EPA in *T. vaginalis* strains Casu2 and ATCC 50142, respectively. In contrast to EPA, concentrations higher than 380 μM DHA (**Fig. 2b** and **2e**) and 760 μM AA (**Fig. 2c** and **2f**) were required to kill >90% of the parasite population.

T. vaginalis isolates ATCC 50142 and Casu2 were also exposed to a range of concentrations of oxidized PUFAs, namely oxEPA, oxDHA and oxAA, in a MIC assay (**Fig. 3**). Our results show that of all three oxidized PUFAs, oxEPA exhibited the least detrimental effect, requiring > 3 mM oxEPA to cause a decrease in viability in both strains of *T. vaginalis* at 24 h (**Fig. 3a** and **3d**). For instance, 3 mM oxEPA resulted in 25.8 ± 13.1% and 83.3 ± 8.3% viability at 24 h for Casu2 and ATCC 50142 parasites, respectively. Using oxDHA, lower concentrations (> 380 μM oxDHA) were effective in killing the Casu2 and ATCC 50142 parasites (**Fig. 3e**). Of the three oxidized PUFAs, oxAA demonstrated the best anti-parasitic activity (> 380 μM oxAA) against both Casu2 and ATCC 50142 species (**Fig. 3c** and **3f**). Overall, the greatest contrast in activity of the three PUFAs examined was observed using EPA, with the native form exhibiting the most potent anti-parasitic activity, and the oxidized form displaying the least.

The effect of oxidized EPA on T. vaginalis growth

Incubation of *T. vaginalis* with oxEPA led to increased motility of the parasite compared to control samples, and since the parasites were resistant to oxEPA at concentrations < 3 mM, we explored the hypothesis that lower concentrations of oxEPA may be protective. Thus, we obtained growth curves for *T. vaginalis* isolates ATCC 50142 and Casu2 in the presence and absence of oxEPA (10 μM) (**Fig. 4**). The results show that the initial growth rates of the Casu2 and ATCC 50142 parasites grown in the presence of oxEPA did not vary significantly compared to control samples. However, parasites grown in the presence of oxEPA statistically achieved greater density at time points ≥ 20 h compared to controls.

Mass spectrometric analysis of oxidized EPA

The composition of native EPA compared to oxEPA (*i.e.* EPA sodium salt oxidized in air for three days) was analyzed by negative mode mass spectrometry (**Fig. 5**). Native EPA shows a parent ion at 300.9 m/z, which corresponds to the EPA anion (exact mass of EPA^- = 301.2 amu)

(**Fig. 5a**). The natural isotopes of EPA give rise to shoulders on the main peak at 301.7 and 302.7 amu. All manipulations involving native EPA prior to mass spectrometric analysis were performed under nitrogen gas to prevent any deliberate oxidation. Despite these precautions, we observed some oxidized products at 316.8 m/z and 332.9 m/z, reflecting the incorporation of one and two oxygen atoms, respectively (black spectrum, **Fig. 5b**). These results may indicate that the native EPA sodium salt solid was received in this state, or else some spurious autoxidation occurred during the handling process, which could not be controlled. This observation, however, was reproducible.

Mass spectral analysis of oxEPA shows a parent ion peak at 300.9 m/z (red spectrum, **Fig. 5a**) that is reduced in intensity compared to native EPA (black spectrum, **Fig. 5a**), and also the presence of several clusters of peaks in the 310–410 m/z region that are centered at 316.8, 322.9, 348.8, 364.8, 380.5, and 396.8 m/z (red spectrum, **Fig. 5b**). The average separation between the main peaks in these clusters is 16.0 ± 0.1 amu and thus represents the addition of one, two, three, four, five and six oxygen atoms to the EPA anion, respectively (red spectrum, **Fig. 5b**).

DISCUSSION

Effective treatment for trichomoniasis, caused by the parasite *T. vaginalis*, includes metronidazole [2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol], but since metronidazole-resistant strains are known (Robinson 1962; Krajden et al. 1986; Cudmore et al. 2004), it is important to have other therapies available for treatment of this parasite. In recent decades, the search for natural products that promote healthy outcomes has increased. Literature reports indicate that EPA not only provides benefits in humans (De Caterina and Basta 2001), but also displays anti-parasitic activity (Kumaratilake et al. 1992). In our study, an investigation of the effects of EPA on both metronidazole-sensitive (Casu2) and -resistant (ATCC 50142) strains of *T. vaginalis* survival yielded similar results, although the effect observed depended on whether native or oxidized forms of EPA sodium salt (denoted as EPA) were used. Native EPA limited growth and caused parasite death, while oxEPA showed evidence of increased cell density and motility compared to control samples.

Using ATCC 50142 parasites, we observed that 10 μM EPA caused approximately 50% death at 24 h, which was significant compared to control, whereas 100 μM completely killed the parasite at 48 h under both aerobic and anaerobic conditions (**Fig. 1**). Notably, native EPA displayed anti-parasitic activity in the presence and absence of oxygen, indicating that the mechanism by which EPA causes cell death does not rely on reacting with dissolved oxygen and scavenging it from the surrounding medium.

Metronidazole is thought to be reduced by low redox potential iron-sulfur proteins in the trichomonad hydrogenosomes, producing a series of nitro radicals that disrupt the DNA of microbial cells, although the mechanism of metronidazole activation is not completely understood (Muller 1993; Land et al. 2004; Leitsch et al. 2009). Clinical isolates exhibiting metronidazole tolerance have defective oxygen scavenging properties (Ellis et al. 1994), which leads to quenching of the nitro radical (Lloyd and Pedersen 1985). Thus, metronidazole-resistant

strains of *T. vaginalis* are especially resistant under aerobic conditions. In this regard, it has previously been reported that the mean aerobic and anaerobic susceptibilities at 48 h of metronidazole-resistant strains, including ATCC 50142, is 195.5 μg/mL (1.14 mM) and 5.05 μg/mL (29.5 μM) metronidazole, respectively (Lossick et al. 1986; Muller et al. 1988). In our study, native EPA inactivated *T. vaginalis* ATCC 50142 in the presence and absence of oxygen (**Fig. 1**), indicating that EPA kills the parasite by a different mechanism compared to metronidazole.

To determine whether other long-chain PUFAs exhibit a similar effect to EPA, we investigated the anti-parasitic activities of AA and DHA, along with EPA, in their native and oxidized forms, against Casu2 and ATCC 50142 strains in a MIC assay. AA (20:4 ω-6) is structurally similar to EPA (20:5 ω-3), containing a similar number of carbon atoms, but with one less double bond. DHA (22:6 ω-3), like EPA, is an omega-3 fatty acid, but is longer than EPA (and AA) by two carbon atoms, and has six double bonds. In humans, products of omega-3 fatty acids (EPA and DHA) are associated with anti-inflammatory properties (Serhan et al. 2000; Oh et al. 2011) and interfere with the arachidonic acid (AA) cascade that produces eicosanoids with pro-inflammatory activities (Laneuville et al. 1995; Achard et al. 1997).

Of the three native PUFAs evaluated, native EPA showed the best anti-parasitic activity. Our results indicated that approximately 90% inhibition of parasite activity at 24 h could be achieved using 190 μM EPA (60 μg/mL) and 380 μM EPA (120 μg/mL) for the Casu2 and ATCC 50142 strains, respectively (**Fig. 2**). At 48 h, these levels were reduced to 25% of the original values, at 48 μM (15 μg/mL) EPA and 96 μM (30 μg/mL) EPA, respectively, for Casu2 and ATCC 50142. Thus, the ATCC 50142 strain required approximately twice the amount of native EPA than the Casu2 strain. Native DHA was less effective than EPA requiring up to 760 μM DHA to cause parasite death. Native AA was the least effective of the three native PUFAs examined, requiring concentrations in excess of 760 μM AA to cause cell death. The exact mechanism by which EPA limits parasite growth is unknown, but its incorporation may lead to the modulation of gene expression or the activation of harmful signaling pathways (Russell and Burgin-Maunder 2012).

While native EPA causes parasite inactivity, our results indicate that oxEPA was completely ineffective in causing parasite death requiring levels that are >3 mM oxEPA (**Fig. 3**). In comparing the anti-parasitic activity of oxEPA with that of oxDHA and oxAA, it is apparent that oxEPA is the least toxic. Also, oxAA was slightly more effective than native AA in causing parasite death. These results indicate that small changes in structure can completely alter the activity of a lipid molecule and highlights the need for a better evaluation of fatty acid-related compounds as potential drug candidates and elucidation of their mechanism of action.

The fact that oxEPA was ineffective in causing cell death prompted us to evaluate whether oxEPA could be beneficial to the parasite. Using low levels (10 μM), we observed that oxEPA promoted the growth of denser populations of *T. vaginalis*, suggesting the possibility that certain oxygenated forms of EPA may, in fact, be beneficial to the parasite (**Fig. 4**).

Native EPA autoxidizes readily in air, *via* non-enzymatic pathways, leading to the observation by ESI-MS of different oxidized products containing one to six additional oxygen

atoms compared to EPA, with various degrees of bond saturation (**Fig. 5**). Thus, mass spectral results indicate that the composition of oxEPA is complex, and several candidates may be responsible for stimulating the proliferation and motility of *T. vaginalis.* These species are not formed during the mass spectrometric process, as we previously demonstrated that they arise in a time-dependent manner (Jordan and Upmacis 2013). More recently, using desorption electrospray ionization-mass spectrometry (DESI-MS) to analyze oxidized EPA, up to 6 oxygen atoms were also noted, representing three peroxidations of EPA (West et al. 2014). Our mass spectral results monitoring the autoxidation of EPA sodium salt following three days in air are consistent with our previous observations characterizing the autoxidized products of EPA sodium salt at two and four days (Jordan and Upmacis 2013). Many isomers can result from oxygen addition, since initial hydrogen abstraction can occur at several bis-allylic positions between the double bonds of EPA (*i.e.* C-7, C-10, C-13 and C-16) (Jordan and Upmacis 2013). These products may contain different combinations of hydroxyl, keto and peroxyl moieties, although compounds bearing dioxolane rings or cyclic peroxides are also possible (Yin et al. 2007; West et al. 2014).

Our mass spectral data indicate that the oxidation products that mainly differ from native EPA possess ≥ 3 oxygen atom additions compared to EPA, and potentially include formulas that are consistent with trihydroxyeicosapentaenoic acid isomers, $C_{20}H_{30}O_5$, also known as EPA-derived or E-series resolvins (Serhan et al. 2002). Autoxidation pathways that lead to the production of these species are given elsewhere (Jordan and Upmacis 2013). Thus, the higher mass species within the complex oxEPA mixture, which are absent in native EPA, may be responsible for the increased density observed.

While speculative, these results may indicate that host derived oxidized lipid products may modulate parasite survival. Notably, aspirin-treatment, which leads to the production of E-series resolvins (Serhan et al. 2000; Oh et al. 2011), increased the survival of *Trypanosoma cruzi* in mice with Chagas disease (Mukherjee et al. 2011). In addition, *Candida albicans,* an opportunistic fungal pathogen of humans, has been shown to biosynthesize resolvins that modulate the host immune response (Haas-Stapleton et al. 2007). These reports highlight the fact that the types and actions of bioactive lipids that modulate parasitic growth and activity are not well understood.

Only a few studies have investigated the role of lipid metabolism in *T. vaginalis* (Beach et al. 1990; Beach et al. 1991; Guschina et al. 2009; Singh et al. 2009). *T. vaginalis* can incorporate AA and DHA (Beach et al. 1990; Shaio et al. 1992) but, to date, there are no reports examining the effects of EPA on this parasite. Notably, uptake of AA by *T. vaginalis* leads to an increased production of leukotriene B_4, indicating that *T. vaginalis* has the ability to metabolize AA (Shaio et al. 1992; Nam et al. 2011; Nam et al. 2012). It is possible that oxAA interferes with this pathway, leading to the observation in our study that oxAA is more detrimental to *T. vaginalis* survival than native AA.

In summary, we have found that native and oxidized PUFAs can have different effects on *T. vaginalis* activity. In particular, native EPA has a deleterious effect on the parasite, whereas the presence of oxEPA was associated with an increased density of the parasite. In this regard, our data suggest that the parasite may possess pathways that readily distinguish between native

EPA and oxidation products of EPA. Lipid metabolism in *T. vaginalis*, however, has not been well-studied. A better understanding of the structure and function of lipids in *T. vaginalis* may facilitate the design of synthetic fatty acids that incorporate the optimum structure requirements that are effective for the treatment of metronidazole-resistant *T. vaginalis.*

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FIGURE LEGENDS

Figure 1: **The toxic nature of native EPA is not dependent on aerobic conditions.** *Trichomonas vaginalis* parasites (strain ATCC 50142) were incubated under (a) aerobic and (b) anaerobic conditions with EPA (10 and 100 μM). Motile parasites were counted at 24 and 48 h. (a) By one-way ANOVA, a significant difference was observed between the groups under aerobic conditions at 24 h and 48 h ($p <$ 0.0001 at both time points). Compared to control, and using Tukey's Multiple Comparison post-hoc test, 10 μM and 100 μM EPA significantly decreased parasite counts at 24 h (*, $p < 0.05$ and < 0.001, respectively), and at 48 h (*, $p <$ 0.001 in both cases). 100 μM EPA also significantly diminished parasite counts compared to the lower dose of 10 μM EPA at 24 h and 48 h (#, $p < 0.01$ and $<$ 0.05, respectively). (b) By one-way ANOVA, a significant difference was observed between the groups under anaerobic conditions at 24 h and 48 h ($p =$ 0.0027 and 0.0001, respectively). Compared to control, and using Tukey's Multiple Comparison post-hoc test, 10 μM and 100 μM EPA significantly decreased parasite counts at 24 h (*, $p < 0.01$ and < 0.05, respectively), and at 48 h (*, $p < 0.01$ and < 0.001). 100 μM EPA significantly diminished parasite counts compared to the lower dose of 10 μM EPA at 48 h (#, $p < 0.05$). (c) A ratio of parasite counts observed under aerobic versus anaerobic conditions.

Figure 2: **Anti-parasitic activity of native EPA, DHA and AA.** Anti-*T. vaginalis* activity of native EPA, DHA and AA against isolates Casu2 (a)–(c), and ATCC 50142 (d)–(f). All determinations were performed in triplicate. The data are represented as the mean and the error bars indicate SEM values.

Figure 3: **Anti-parasitic activity of oxidized EPA, DHA and AA.** Anti-*T. vaginalis* activity of oxEPA, oxDHA and oxAA against isolates Casu2 (a)–(c), and ATCC 50142 (d)–(f). All determinations were performed in triplicate. The data are represented as the mean and the error bars indicate SEM values.

Figure 4: ***Oxidized EPA enhances the growth of T. vaginalis.*** The effect of oxEPA on the growth curves of *T. vaginalis* strains (a) Casu2, and (b) ATCC 50142. All determinations were made in triplicate; error bars indicate ± SEM values.

Figure 5: **A mass spectrum of oxidized EPA reveals products containing one to six additional oxygen atoms.** Mass spectra showing a comparison of native EPA sodium salt (black spectrum) and EPA sodium salt oxidized in air for three days (red dotted spectrum) in the (a) 295–310 m/z (amu) range showing the parent ion at 300.9 m/z (amu) and in the (b) 310410 m/z (amu) range displaying clusters of ions with the mass of the central peak of each group corresponding to 1, 2, 3, 4, 5 or 6 oxygen [O] atom additions to EPA. Peaks either side of the dominant ion in each cluster represent a difference of 2.06 ± 0.04 amu. Possible molecular formulas are reported for the neutral oxidized fatty acid species.

Figure 1

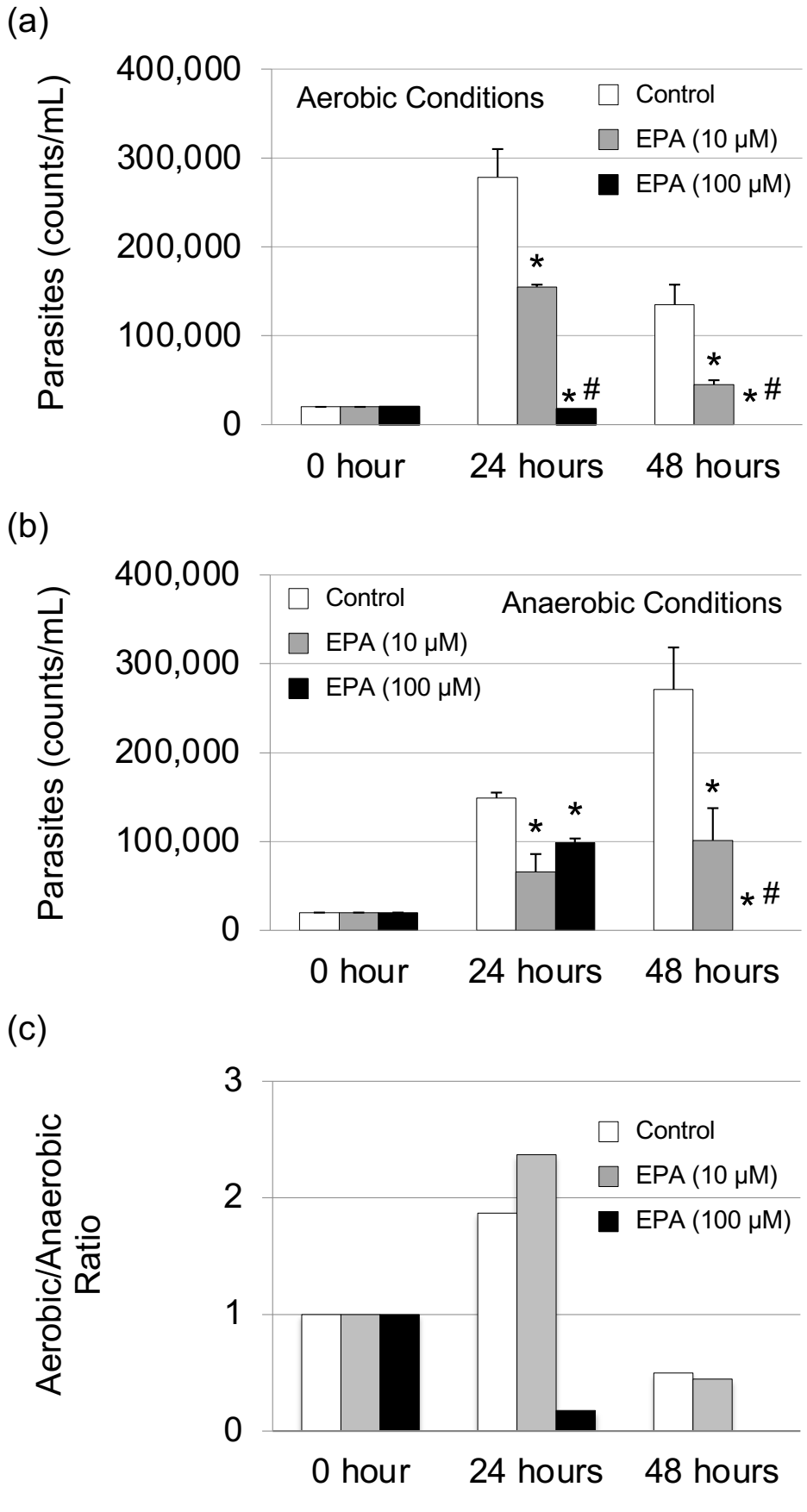

(a)
Parasites (counts/mL)
400,000
300,000
200,000
100,000
0
Aerobic Conditions
Control
EPA (10 μM)
EPA (100 μM)
*
*#
*
*#
0 hour
24 hours
48 hours
(b)
Parasites (counts/mL)
400,000
300,000
200,000
100,000
0
Control
EPA (10 μM)
EPA (100 μM)
Anaerobic Conditions
*
*
*
*#
0 hour
24 hours
48 hours
(c)
Aerobic/Anaerobic Ratio
3
2
1
0
Control
EPA (10 μM)
EPA (100 μM)
0 hour
24 hours
48 hours

Figure 2

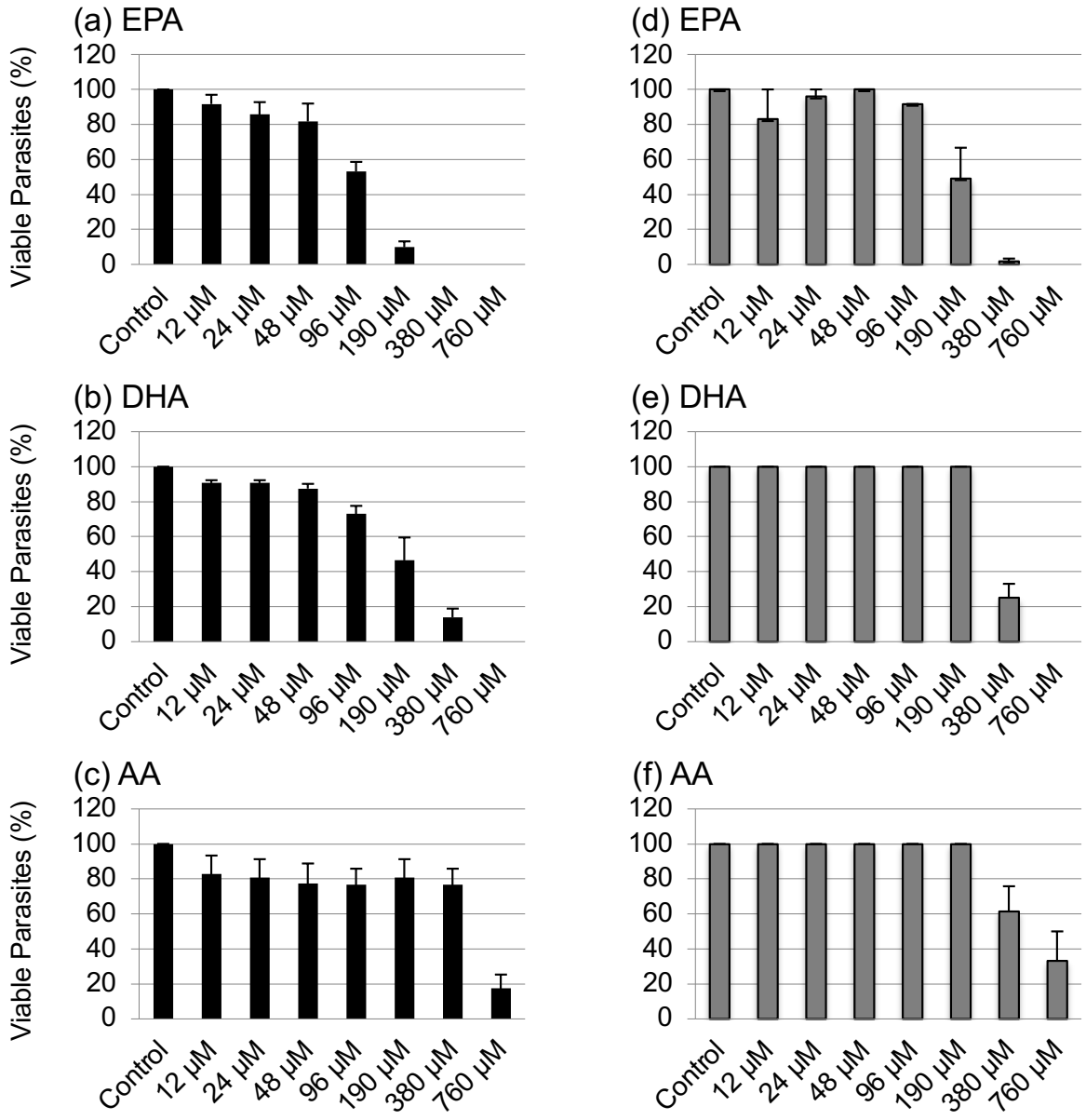

(a) EPA
(b) DHA
(c) AA
(d) EPA
(e) DHA
(f) AA
Viable Parasites (%)
120
100
80
60
40
20
0
Control
12 μM
24 μM
48 μM
96 μM
190 μM
380 μM
760 μM

Figure 3

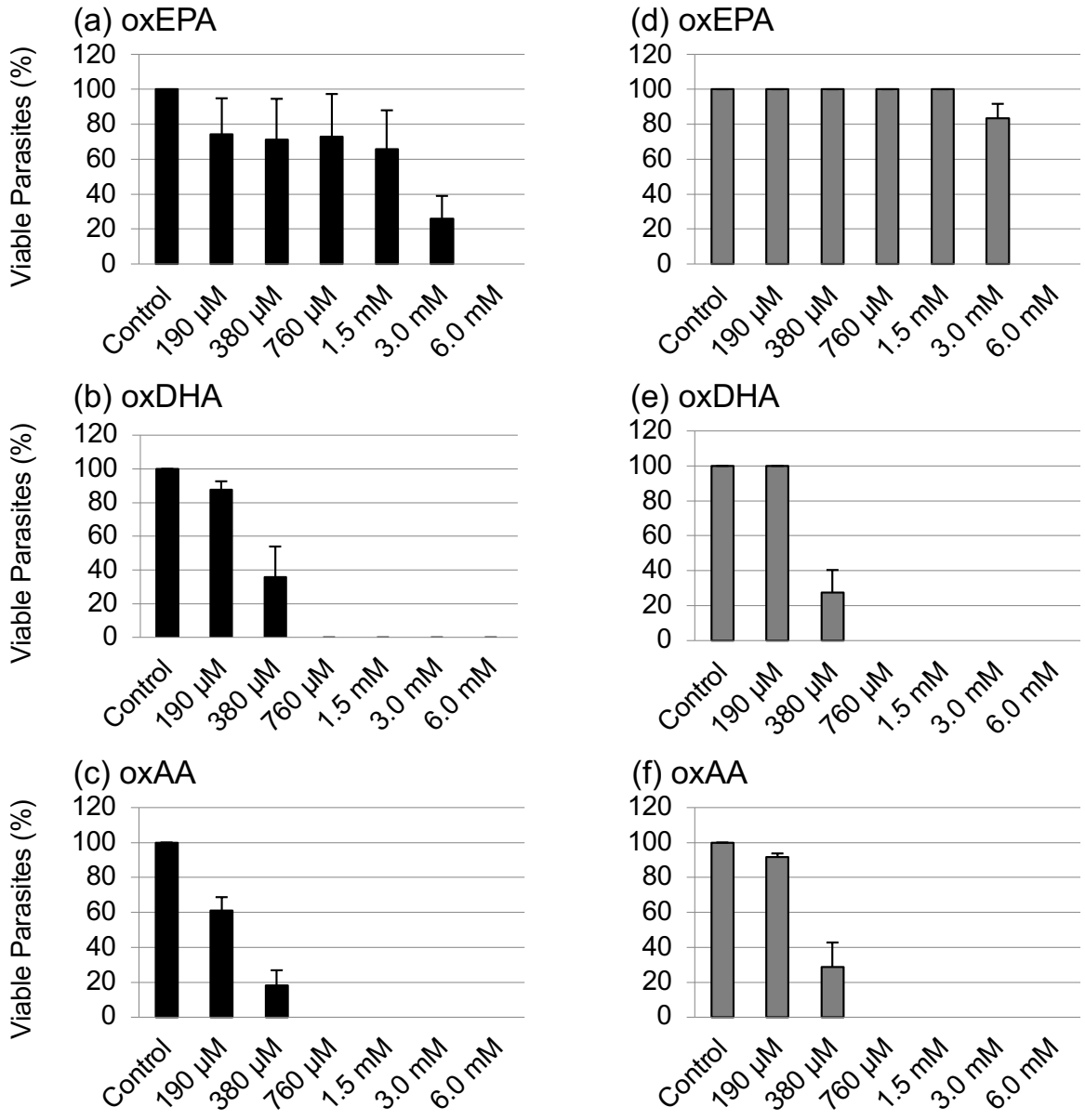
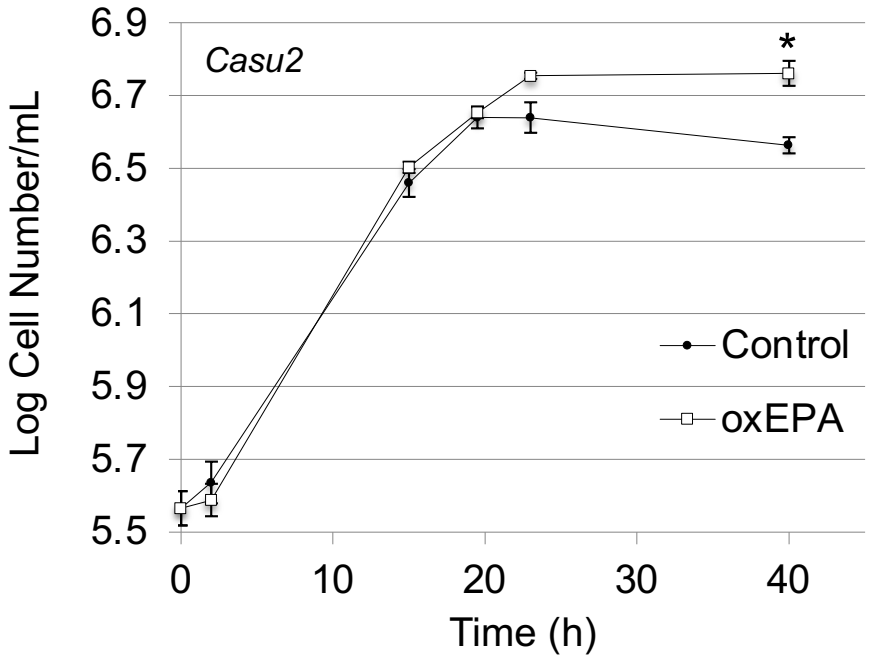

(a) oxEPA
(b) oxDHA
(c) oxAA
(d) oxEPA
(e) oxDHA
(f) oxAA
Viable Parasites (%)
120
100
80
60
40
20
0
Control
190 μM
380 μM
760 μM
1.5 mM
3.0 mM
6.0 mM

Figure 4

(a)


Casu2
*
Log Cell Number/mL
6.9
6.7
6.5
6.3
6.1
5.9
5.7
5.5
0
10
20
30
40
Time (h)
Control
oxEPA

(b)

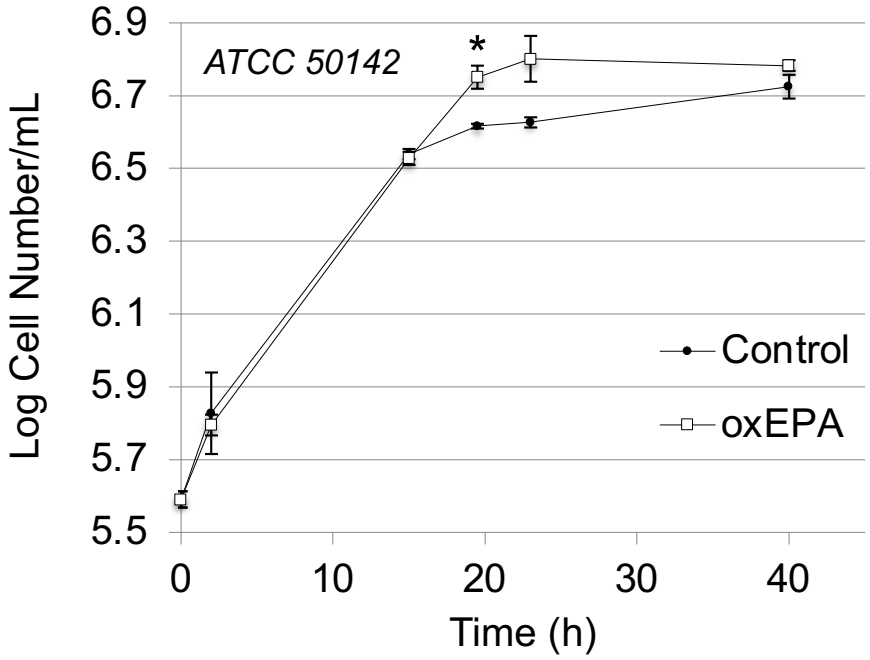
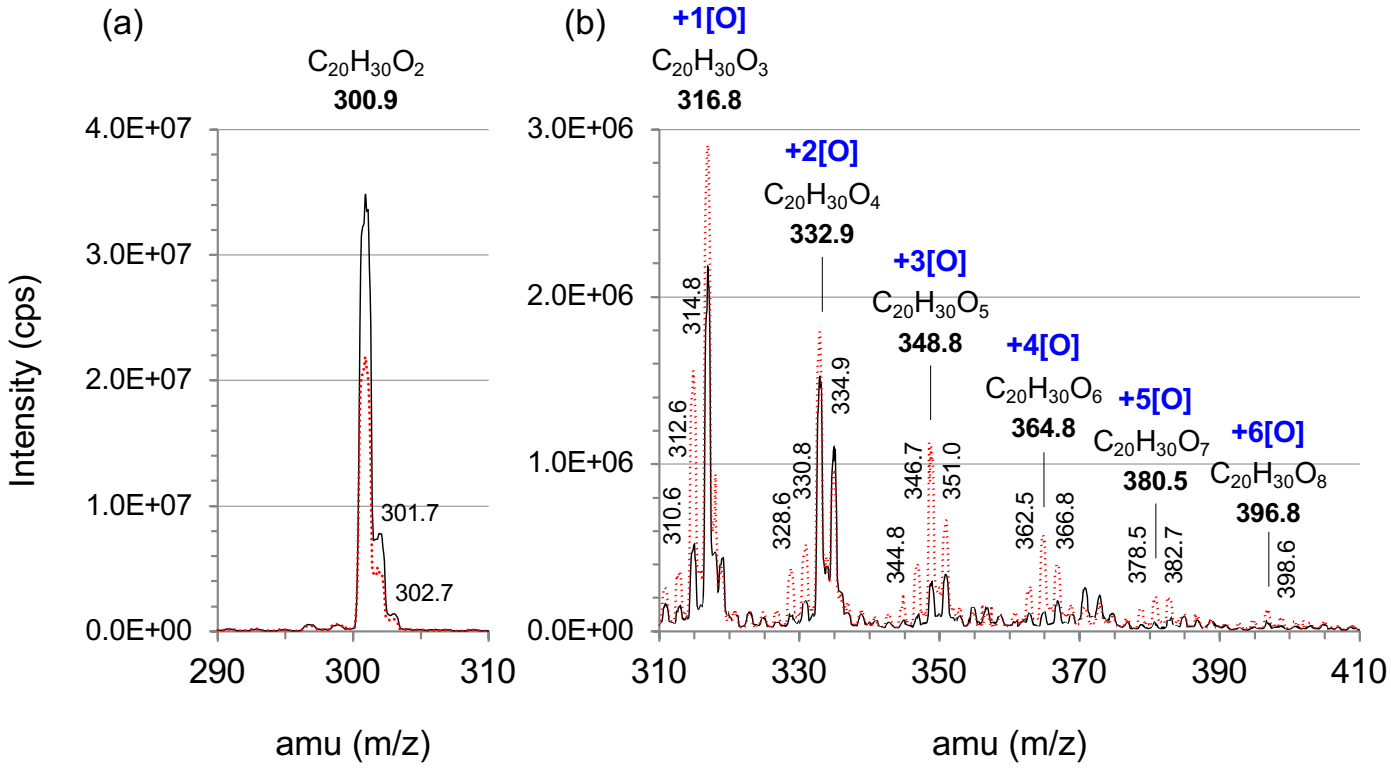

ATCC 50142
*
Log Cell Number/mL
6.9
6.7
6.5
6.3
6.1
5.9
5.7
5.5
0
10
20
30
40
Time (h)
Control
oxEPA

Figure 5


(a)
C20H30O2
300.9
4.0E+07
3.0E+07
2.0E+07
1.0E+07
0.0E+00
301.7
302.7
290
300
310
amu (m/z)
Intensity (cps)
(b)
+1[O]
C20H30O3
316.8
+2[O]
C20H30O4
332.9
+3[O]
C20H30O5
348.8
+4[O]
C20H30O6
364.8
+5[O]
C20H30O7
380.5
+6[O]
C20H30O8
396.8
3.0E+06
2.0E+06
1.0E+06
0.0E+00
310.6
312.6
314.8
328.6
330.8
334.9
344.8
346.7
351.0
362.5
366.8
378.5
382.7
398.6
310
330
350
370
390
410
amu (m/z)